

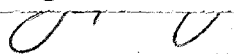
AN ABSTRACT OF THE DISSERTATION OF

Ying Xiao for the degree of Doctor of Philosophy in Wood Science
presented on February 23, 2004.

Title: Application of Green Fluorescent Protein (GFP) for Studying Interactions between *Ophiostoma piceae* and *Trichoderma harzianum* in Freshly Sawn Douglas-Fir Sapwood.

Abstract approved:

Signature redacted for privacy.



Jeffrey J. Morrell

While microbial colonization of wood is presumed to be characterized by a myriad of interactions between numerous organisms, studying these processes is often difficult owing to the opaque nature of the wood and the inability to readily distinguish among the many species colonizing the material. One method for enhancing the ability to distinguish organisms is to induce specific proteins in one or more organisms that can be detected using fluorescence or other light microscopic techniques. The insertion of genes for the production of green fluorescent proteins produced by the jellyfish, *Aequora victoria*, has been widely used to visualize a variety of organisms. In this study, an important wood sapstain fungus, *Ophiostoma piceae*, and its biocontrol agent, *Trichoderma harzianum*

were transformed using a green fluorescent protein (SGFP) gene under the control of the *ToxA* promoter of *Pyrenophora tritici-repentis*. Spore germination and growth developments of these fungi on freshly sawn Douglas-fir sapwood were examined using fluorescence microscopy. The expression of *gfp* was particularly useful for studying the spatial distribution of young hyphae in wood.

The *gfp* transformants were used to study interactions between *T. harzianum* and *O. piceae* in freshly sawn Douglas-fir sapwood. *O. piceae* growth decreased with increasing spore ratios of *T. harzianum* to *O. piceae*. Prior establishment of *T. harzianum* was effective against *O. piceae* growth on Douglas-fir sapwood, but killing established *T. harzianum* by γ -irradiation eliminated this effect. Killing *T. harzianum* by autoclaving after prior establishment afforded partial protection against *O. piceae* growth. The results illustrate the potential role of active growth in biocontrol against stain fungi.

Application of Green Fluorescent Protein (GFP) for Studying Interactions between
Ophiostoma piceae and *Trichoderma harzianum* in Freshly Sawn Douglas-Fir
Sapwood

by

Ying Xiao

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Doctor of Philosophy thesis of Ying Xiao presented on February 23, 2004.

Approved:

Signature redacted for privacy.

Major Professor, representing Wood Science

Signature redacted for privacy.

Head of Department of Wood Science and Engineering

Signature redacted for privacy.

Dean of Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Ying Xiao, Author

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Application of Green Fluorescent Protein (GFP) for Studying
Interactions between *Ophiostoma piceae* and *Trichoderma harzianum*
in Freshly Sawn Douglas-Fir Sapwood

Chapter 1: General Introduction and Literature Review

General Introduction and Literature Review

GENERAL INTRODUCTION

Wood is one of our only truly renewable natural resources. Wood-based products are very important materials that have been widely used in construction. However, wood is composed of organic matter, and is prone to microbial attack. Sapstain fungi can rapidly colonize and discolor sapwood, reducing the value of wood products and causing millions of dollars in annual losses to the wood industry. The primary approaches to controlling fungal discoloration have been kiln drying or chemical treatment shortly after sawing. However, increasing environmental concerns have led to a search for alternative methods. One alternative to chemical treatment is biological control (biocontrol): using one organism to control the detrimental activities of another. Biocontrol is not a new concept; fungi have been employed for this purpose on a variety of wood-based materials since 1963, but there have been few broadly successful applications against stain and mold fungi. The failures have highlighted the need for more fundamental research on *in situ* microbial interactions between biocontrol agents and their intended targets.

An important aspect of this need is the ability to study microbial interactions within wood. The opaque nature of wood makes it difficult to observe interactions more than a few cells from the surface. Wood can be cut into thin sections, but the structural similarity in fungal hyphae of different species makes it difficult to determine which fungus is present. A number of fluorescent probes are available that are specific for components in fungal hyphae, but most of these react with hyphal components common to many fungi.

The green fluorescent protein (GFP) technique inserts a *gfp* gene originally isolated from a jellyfish, *Aequora victoria*, into a microorganism. GFP has been successfully used as a biomarker in many microorganisms which are transformed with the *gfp* genes to produce distinct fluorescence. Transformation of fungi with *gfp* genes has proven to be useful for understanding microbe-plant interactions and the fundamental aspects of biocontrol. In this study, we transformed an economically important sapstain fungus, *Ophiostoma piceae* (Münch) Syd. & P. Syd., and its potential biocontrol agent, *Trichoderma harzianum* Rifai (a mold fungus). The transformants were then used to study interactions between the two fungi. Implications of these studies on improving biocontrol efficacy are also discussed.

LITERATURE REVIEW

Application of *Trichoderma* spp. in Biocontrol of Wood Degrading Microorganisms

Biocontrol uses one organism to control the detrimental activities of another. *Trichoderma harzianum* has proven effective as a biocontrol agent against a range of important airborne and soilborne plant pathogen, and wood degrading fungi (Rishbeth, 1963; Shields and Atwell, 1963; Ricard, 1976; Chet, 1987; Seifert *et al.*, 1988; Morrell and Sexton, 1990; Behrendt *et al.*, 1995; McAfee and Gignac, 1996; Ejechi, 1997; Kay, 1997; Sutherland and Brasier, 1997; Score *et al.*, 1998; Brown and Bruce, 1999; Brown *et al.*, 1999; Schoeman *et al.*, 1999; Smouse *et al.*, 1999; Yang and Rossignol, 1999; McAfee *et al.*, 2001; Vanneste *et al.*, 2002). However, there has been little commercial success with biocontrol of wood degrading microorganisms (Freitag *et al.*, 1991), although *Trichoderma* spp. have found some commercial uses in the control of tree pathogens (Ricard and Highley, 1988 a&b).

One factor that hinders commercialization of biocontrol has been the large difference between laboratory and field performance of *Trichoderma* spp. While wood material colonized by *Trichoderma* spp was decay resistant in soil block tests, uneven colonization of *Trichoderma* spp. in creosoted poles resulted in biocontrol failure in field trials (Bruce and King, 1986 a&b, Bruce *et al.*, 1989; Bruce *et al.* 1990). *T. polysporum* and *T. harzianum* performed well against

several brown-rot fungi in loblolly pine or Douglas-fir out of ground contact, but had little effect when wood was exposed to soil (Morrell and Sexton, 1990). These two biocontrol agents showed little effect on white-rot fungi in the same trial.

Most biocontrol agents perform inconsistently due to uncontrollable environmental factors, such as temperature, moisture content, type and aging of the substrate, and the presence of other microorganisms. There is a critical lack of understanding of the factors that affect *T. harzianum* and its target organisms.

T. harzianum is believed to function as a biocontrol agent via combinations of three mechanisms: competition for nutrients, antibiosis, or actions by cell-wall lytic enzymes (Dennis and Webster, 1971 a-c; Chet, 1987; Papavizas, 1985). However, most studies have been performed on solid artificial media or in liquid cultures supplemented with different carbon sources, and the ability of this fungus to function via these strategies has not been fully tested *in vivo*. Growth medium can play a crucial role in the effectiveness of *Trichoderma* spp., reflecting the complexity of microbial interactions *in situ* (Doi and Yamada, 1992; Srinivasan *et al.*, 1992; Score and Palfreyman, 1994; Score *et al.*, 1998). Therefore it is important to use wood as the substrate in interaction studies. One problem with wood is the inability to resolve individual microorganisms in wood. This limitation prevents effective examination of fungal interactions in wood.

Competition for nutrients makes it important for organisms to develop systems capable of sequestering nutrients more efficiently than their competitors. Siderophores are iron-regulated, low-molecular weight ferric specific chelators

that are produced by wood decay fungi and *Trichoderma* spp. (Jellison *et al.*, 1990; Anke *et al.*, 1991). Studies of the potential role of siderophores in the biocontrol of wood decay fungi using *Trichoderma* spp. suggested that basidiomycete inhibition by *Trichoderma* spp. was due to siderophore competition for iron, an essential element for fungal growth (Srinivasan *et al.*, 1995).

Some fungi also produce volatile and non-volatile metabolites that can inhibit competing fungi. Metabolites obtained from *T. harzianum* have antifungal properties that inhibit wood degrading fungi (Bruce *et al.*, 1984; Claydon *et al.*, 1987; Bettucci *et al.*, 1988; Avent *et al.*, 1992; Srinivasan *et al.*, 1992; Horvath *et al.*, 1995; Bruce *et al.*, 1996; Hill *et al.*, 1997). These metabolites could be exploited for biocontrol purposes but it is unclear how these substances are produced *in vivo*.

Mycoparasitism via production of lytic enzymes that degrade fungal cell walls has been described as the mechanism of action of *Trichoderma* species in the biological control of commercially important plant pathogens. Constitutive chitinases may partially degrade the cell walls of fungi, thereby generating oligosaccharides containing chitin (N-acetyl- β -D-Gluocosamine) that may act as elicitors for the general antifungal response of *Trichoderma* (Lora *et al.*, 1994). Zellinger *et al.* (1999) demonstrated that a gene encoding the CHIT42 endochitinase was expressed prior to physical contact of the fungus with its host, thus indicating that this enzyme could be involved in the very early stages of the

mycoparasitism by *T. harzianum*. However, little information is available on the significance of this mechanism for the biocontrol of wood inhabiting fungi.

Freitag and Morrell (1992) measured levels of activities of selected wood degrading enzymes in pure or mixed cultures of *T. harzianum* and a white-rot fungus, *Trametes versicolor*. Laccase activities of *T. versicolor*, and to a lesser extent, cellobiase activities of both fungi were significantly increased in mixed cultures. Specific cellobiase, specific cellulase and peroxidase activities were enhanced in low-N media. Nitrogen level also increased the production of specific chitinase and laminarinase by *Trichoderma* spp. in liquid cultures (Bruce *et al.*, 1996). Large interstrain and interspecies differences exist in the levels of production of both enzymes.

The presence of microorganisms in the same environment can also affect biocontrol activity. The ability of *T. hamatum* to suppress *Rhizoctonia* damping-off in a bark compost-amended container medium was reduced significantly by the presence of some thermophilic fungi (Chung and Hoitink, 1990). Microbiota in untreated soil (Bae and Knudson, 2000) and a fungal-feeding nematode (Bae and Knudson, 2001) reduced growth of *T. harzianum* in soil and may significantly constrain the biocontrol efficacy.

Another *Trichoderma* species, *T. viride*, inhibited decay in three tropical timbers by a white-rot fungus and a brown-rot fungus when inoculated on wood simultaneously with the decay fungi (Ejechi and Obuekwe, 1994). Fungal attack was enhanced by the prior growth of *T. harzianum* before sterilization of wood.

This enhancement did not occur, however, if the wood was extracted before initial exposure to *T. harzianum*. These observations suggested that prior growth of *T. harzianum* reduced the decay retarding property of wood extractives. In a follow up study by the same authors, the significance of pH manipulation on biocontrol efficacy of *T. harzianum* was studied. *T. harzianum* grew better as the pH approached neutral while the reverse was true for the wood decay fungi (Ejechi and Obuekwe, 1996). The biological control agent was lethal to the decay fungi when pH was favorable.

The presence of viable *Trichoderma* spp. provided the best protection to wood, although the presence of non-viable *Trichoderma* spp. (wood irradiated after *Trichoderma* spp. growth) also afforded some protection, suggesting that biocontrol is a function of more than one mechanism (Score et al., 1998).

Wood may also play a critical role in biocontrol efficacy because the ability of fungi to compete with each other within the host may depend on viability (Stone and Simpson, 1991). Field studies showed that gamma-irradiated and freshly sawn Corsican pine timber had different susceptibility to colonization by mold and sapstain fungi (Williams et al., 1998). *T. harzianum* grew much faster in irradiated wood, while *O. piceae* grew similarly in fresh and irradiated wood. When both fungi were applied as a mixed spore suspension, *T. harzianum* strongly outcompeted *O. piceae* on irradiated wood, but the outcome was reversed in fresh wood. In a parallel study, Scots pine log billets stored for up to nine weeks were naturally aged or sterilized by autoclaving or irradiation, and then inoculated with

sapstain fungi (Strong *et al.*, 1998). Growth rates were highest in freshly felled logs which had been sterilized by autoclaving or irradiation. Both autoclaving and gamma irradiation kill any living cells in the substratum, including both ray parenchyma and any established microorganism, and therefore destroy any potential host response of the wood to invading fungi. These processes also alter the physical and biochemical status of the wood. *O. piceae* appeared to be more tolerant of changes associated with ageing/drying timber than two other sapstain fungi. The ability to tolerate more stressful environments may increase the competitive ability of *O. piceae*.

Although three main mechanisms have been proposed for *Trichoderma* spp., different strategies may be employed by various *Trichoderma* species against a given microorganism. Identifying the main mode of action by a biocontrol agent could be used to improve biocontrol efficacy.

Applications of GFP in Filamentous Fungi and Plant-Microbe Interactions

The jellyfish *Aequorea Victoria*, *gfp* gene and its mutated forms have been successfully expressed in bacteria, yeast, mammals, plants, and filamentous fungi since its first cloning and expression (Prasher *et al.*, 1992; Chalfie *et al.*, 1994). Organisms that express *gfp* will produce proteins that fluoresce without any extrinsic factors except UV or blue light and oxygen. Thus, living cells can be observed with minimal perturbation and intercellular activities can be observed directly (Fernández-Ábalos *et al.*, 1998). *In vivo* observation of *gfp* expression is

possible with individual cells, with cell populations, or in whole organisms interacting with symbionts or environments in real time (Finlay, 2000; Lorang *et al.*, 2001). GFP has been widely applied in many biological fields as a biomarker or as a reporter for gene expression. This review will focus on applications in filamentous fungi and microbe-plant interaction.

GFP fundamentally lacks one stage of amplification built into a true enzymatic reporter system in which each protein molecule can generate thousands of chromophore or fluorophore molecules. Because each GFP represents one fluorophore, relatively high levels of GFP expression, as much as 10^6 molecules per cell, may be necessary to give bright signals (Cubitt *et al.*, 1995). High efficiency is therefore desired in GFP transformation. However, the wild-type *A. victoria gfp* gene does not confer appreciable fluorescence to many fungi, primarily because it is not efficiently translated (Spellig *et al.*, 1996; Cormack *et al.*, 1997; Fernández-Ábalos *et al.*, 1998; Maor *et al.*, 1998; Lorang *et al.*, 2001). It is necessary to modify the wild-type *gfp* gene by optimizing its codon usage for high transformation efficiency in different target organisms.

A re-engineered GFP gene sequence (*sGFP* or *sGFP (S65T)*) with the favored codons of highly expressed human proteins can be used in a broad spectrum of transient expression systems (Chiu *et al.*, 1996). These sequences have been completely codon adapted for expression in animal and plant cells (Heim *et al.*, 1995; Sheen *et al.*, 1995; Chiu *et al.*, 1996; Hass *et al.*, 1996). The efficiency of these sequences for transforming filamentous fungi was first

demonstrated by Spellig *et al.* (1996), who transformed a basidiomycete maize pathogen *Ustilago maydis* and obtained high expression and bright fluorescence. Fungal hyphae located intracellularly in plants were clearly detectable. *sGFP* has been the *gfp* gene most often used for transformation of filamentous fungi. *EGFP1* (Clontech, Inc., Palo Alto, Calif.) is another commonly used *gfp* variant for filamentous fungal transformation.

Constitutive expression of *gfp* in a microorganism allows for continuous monitoring of activities, which has proven to be extremely useful for studying plant-microbe interactions.

Suelmann *et al.* (1997) studied nuclear migration and positioning of *Aspergillus nidulans* *in vivo* using GFP. GFP expression plasmids pRS31 and pRS32 containing *sGFP* were constructed to obtain specific expression in nuclei of *A. nidulans*. Epifluorescent images were overlayed with phase contrast views to visualize nuclei and hyphae at the same time. A deleterious effect of the expressed GFP in hyphae was observed under certain illumination conditions. Sivers *et al.* (1999) transformed *A. nidulans* with the GFP plasmid pRS31 (Suelmann *et al.*, 1997) for rapid screening of mutants that had defects in mitosis, nuclear migration and/or hyphal morphology. Mutants with defects in nuclear distribution were also affected in development and/or hyphal morphology, illustrating the interplay of these cell biological processes.

Vanden Wymelenberg *et al.* (1997) transformed *Aureobasidium pullulans* with a self-constructed pTEFEGFP vector containing an *EGFP* gene, the *A.*

pullulans translation elongation factor (TEF) promoter and *Aspergillus awamori* glucoamylase terminator. The transformation allowed quantitative detection of *A. pullulans* transformants on leaf surfaces. Webb *et al.* (2001) also transformed an *A. pullulans* strain with pTEFEGFP vector to study fungal susceptibility to antimicrobial compounds. Fluorescence level in blastospore suspensions of the GFP transformant was directly proportional to the number of viable cells. Therefore GFP fluorescence may be used as a real-time, noninvasive indicator for monitoring cell viability. The loss of fluorescence and cell viability in the presence of biocides might be attributed to the interference of the biocides with intracellular pH regulation.

In their work transforming *A. nidulans*, Fernández-Ábalos *et al.* (1998) constructed a plasmid containing a *sGFP (S65T)* gene to produce soluble GFP that was present throughout the cytoplasm and was able to enter the nucleus. Immunogold staining of hyphae using antibodies against GFP confirmed that GFP was absent from the mitochondria and vacuoles but abundant in the cytoplasm and nuclei. Procedures for extracting cell lysates and fluorimetry were described, and it was shown that the intensity of fluorescence in the protein extract from a given GFP transformant was influenced primarily by the amount of GFP protein produced.

Maor *et al.* (1998) constructed a gGFP vector and a tGFP vector containing *sGFP (S65T)* gene and the *A. nidulans* *Pgdp* promoter to transform *Cochliobolus heterostrophus*, an ascomycete maize pathogen. These vectors were effective in

transforming *Colletotrichum gloeosporioides* f. sp. *aeschynomene* and *Neurospora crassa*, but not *T. harzianum*. GFP accumulation in cytoplasm was observed. The transformed fungus continued to express high levels of GFP 30 days after inoculation, allowing monitoring of fungal growth within the leaf. Fluorescence intensity was also found to be correlated with the amount of protein produced, making it possible to quantify fungal mass and estimate disease levels.

An oomycete, *Phytophthora parasitica* var. *nicotianae*, was transformed with vector pTH210 containing a *sGFP* gene (Bottin *et al.*, 1999) to study *in-vitro* and *in-planta* development. Young hyphae fluoresced almost uniformly, while fluorescence was excluded from vacuoles in older hyphae. Quantitative detection of the parasite *in planta* was also possible through fluorimetry analysis of the extracts from infected tobacco stems. The constitutive amount of SGFP approached 1% of total protein in transformants with the highest transcript accumulation.

van West *et al.* (1999) constructed a pVW2 vector that contained an *egfp* gene to transform *Phytophthora palmivora*. Green fluorescence was detected predominantly in living cells, and was brightest around (or in) the position of nuclei but excluded from the vacuoles. Not all cells of the GFP transformant fluoresced, which may be due to very low expression levels of *gfp* in some cells or the formation of heterokaryotic strains from multi-nucleated protoplasts during transformation. A similar phenomenon was also reported in *Maganaporthe grisea*,

where not all of the appressoria produced by the GFP transformants fluoresced (Xue *et al.*, 2002).

Robinson and Sharon (1999) used the vector developed by Maor *et al.* (1998) to transform a bioherbicide *C. gloeosporioides*. Optimization of the transformation protocol by electroporation of germinated conidia was facilitated by the use of GFP. A similar transformation system was also developed for *gfp* transformation of *Colletotrichum acutatum*, a pathogen that causes anthracnose disease on strawberry (Horowitz *et al.*, 2002). Details of the pathogenic and nonpathogenic lifestyles of *C. acutatum* were determined using GFP-transgenic isolates.

Bae and Knudsen (2000) cotransformed *T. harzianum* with β -glucuronidase (GUS) and GFP genes to monitor growth and activity in natural soils using the vector developed by Vanden Wymelenberg *et al.* (1997). They were able to visualize the transformed *T. harzianum* and determine its growth rate in nonsterile soil, which was impossible when GFP was not available because of the inability to differentiate hyphae from different sources. Lower growth rate of *T. harzianum* in raw soil compared to that in steamed soil was attributed to the influence of the soil microbiota in the untreated soil. The same GFP transformant was used in an interaction study between a fungus-feeding nematode and *T. harzianum*, and showed a positive effect of the nematode on biocontrol efficacy of *T. harzianum*.

A fungal transformation vector, pCT74, that expresses *SGFP* under the control of the *ToxA* gene promoter (Ciuffetti *et al.*, 1997) from *Pyrenophora tritici-repentis* has proven useful for expressing *gfp* in a number of Ascomycota (Lorang *et al.*, 2001).

Lee *et al.* (2002) transformed *O. piceae* strain AU1 and a potential biocontrol agent Cartapip®, an *O. piliferum* albino strain, with the gGFP vector produced by Maor *et al.* (1998). The transformants showed normal phenotypes, growth and colonization on wood. The transformants could be differentiated not only from other species but also from the *O. piliferum* wild-type and albino strains. The authors concluded that GFP could be considered as a useful biomarker and suggested other applications of this technique in the paper. However, these applications were not pursued further (C. Breuil, personal communication).

The use of differently-colored GFPs to track multiple cell types enables differences in cell movement or migration to be visualized in real time without the need to add additional agents or fix or kill the cells. Cowan *et al.* (2000) developed a dual labeling technique involving two GFP variants that fluoresced at wavelengths that are compatible with confocal microscopy. Rodrigues *et al.* (2001) expressed a red fluorescent protein, DsRed, and GFP simultaneously in *Saccharomyces cerevisiae*. Cells of the transformant fluoresced green in the cytoplasm and red, orange, or yellow in the nuclei. The range of nuclei colors may be attributed to colocalization of the two proteins and the differences in the

superposition. Double labeling and confocal laser scanning microscopy (CLSM) allowed tracking the dynamics of the mating process *in vivo*.

Constitutive expression of cyano- (ECFP), green- (EGFP), yellow- (EYFP), and red-fluorescent protein (ERFP) was observed in *Magnaporthe grisea* under the control of the *M. grisea* ribosomal protein 27 promoter, resulting in extremely bright cytoplasmic fluorescence (Czymmek *et al.*, 2002). The attempts to obtain expression of blue-fluorescent protein (EBFP) were unsuccessful with no signal above background autofluorescence detected. Fluorescence was excluded from large organelles such as vacuoles and mitochondria, but accumulated in inter-phase nuclei with ECFP, EGFP, and EYFP. In contrast, ERFP accumulated in vacuoles of many cell types. These results made it possible to simultaneously image combinations of cells expressing different fluorescent proteins after transformed with different genes.

Gage *et al.* (1996) examined the growth and behavior of a GFP transformed *Rhizobia meliloti* strain in alfalfa using confocal laser scanning microscopy (CLSM). Infection threads were seen within individual root hairs as they extended toward the main root body. Sturman *et al.* (2002) developed stable broad-host-range vectors that drove expression of ECFP and EYFP in different rhizobial species. *Rhizobia laguminosarum* strains that expressed ECFP and EYFP, respectively, could be detected unambiguously in a number of different plant species.

Czymmek *et al.* (2002) investigated four-dimensional (4-D) imaging of cell-cell interactions between host and pathogen, adding a time dimension to three-dimensional investigation using CLSM. Continuous imaging of GFP-labeled fungal cells for at least 7h was achievable with laser light attenuated to a very low but sufficient level using an acusto-optic tunable filter without detectable adverse effects on the specimen.

Pöggeler *et al.* (2003) developed a series of versatile reporter plasmids which support the expression of *egfp* gene in filamentous fungi. These vectors have been applied in two ascomycetes, *Acremonium chrysogenum* and *Sordaria macrospora*, to analyze transcription regulation and protein localization and secretion. Cytoplasmic localization using confocal microscopy showed that fluorescence appeared uniformly distributed throughout the cytoplasm in young, fast growing hyphae. GFP fluorescence was not extensively concentrated in the nuclei or in other organelles and appeared to be excluded from the vacuoles.

GFP can be used not only as a biomarker but also as a reporter for gene expression to help identify genes that are responsible for microbial pathogenicity, and thus allows gene manipulation to improve plant resistance against pathogens.

Padgett *et al.* (1996) studied intercellular and intracellular distribution of movement protein (MP) of a virus during infection of leaf tissues using GFP fusion. Subcellular localization of MP:GFP fusion protein in infected leaves was easily visualized. Infected cells were highly fluorescent, reflecting high levels of

activity. In contrast, fluorescence was not detected in cells that were more than two or three cells from those bright fluorescent cells.

Lehmle *et al.* (1997) identified a motor protein required for filamentous growth in *U. maydis* by fusing *sGFP* (Chiu *et al.*, 1996) with a gene that encodes the motor protein. Even distribution of the fusion proteins was observed in both haploid and dikaryotic cells. Basse *et al.* (2002) used *egfp* fusion to localize a *U. maydis* gene in the endoplasmic reticulum.

Berteaux-Lecellier *et al.* (1998) identified a gene, *crol*, required for sexual sporulation of a filamentous ascomycete, *Podospora anserina*. Construction of *crol-egfp* and *crol-sgfp* fusion showed that this gene encodes a cytosolic protein mainly expressed at the beginning of the dikaryotic stage and at the time of ascospore maturation.

Kershaw *et al.* (1998) used GFP as a non-invasive reporter of *MPG1* (a gene encoding a hydrophobin in *M. grisea*) expression. They transformed *M. grisea* with a *MPG1(p)::SGFP* expression vector, pMJK96, containing an *MPG1* promoter and *sGFP* (Chiu *et al.*, 1996). Conidia from GFP transformants were incubated on rice leaves for 24 h. *GFP* expression was clearly observed in appresoria but not in the infection pegs or hyphae, indicating that *MPG-1* expression is restricted to early infection-related development. Aerially borne conidia formed after prolonged incubation showed extensive GFP fluorescence, indicating high *MPG-1* expression during conidiogenesis. Clergeot *et al.* (2001) used GFP fusion to localize a functional gene that encodes a tetraspanin-like

protein in plasma membranes and vacuoles in appressoria. Xue *et al.* (2002) used *egfp* fusion to monitor two fungal virulence genes specifically expressed in appressoria of *M. grisea*. The authors followed the time course of accumulation of the two fusion proteins and compared the expression levels of two genes. Strong fluorescence occurred in appressoria, while there was no detectable fluorescence in well-developed infectious hyphae. Appressoria that produced infectious hyphae lost their fluorescence, suggesting that these fusion proteins were either degraded rapidly or transported into penetration pegs.

Dumas *et al.* (1999) transformed *Colletotrichum lindemuthianum* with *sGFP* (Chiu *et al.*, 1996) to detect expression of an endopolygalacturonase gene, *clpg2*, during bean infection. *GFP* expression under the control of the *clpg2* promoter was monitored at different stages of development of the fungus. High levels of fluorescence were detected in conidia germinating in water on a glass slide when they differentiated an appressorium, although fluorescence was otherwise very weak. Fluorescent germ tubes and swelling appressorium were detected at the surface of infected plants. Fluorescence in germ tubes later decreased whereas fluorescence in the appressorium increased, which may reflect migration of the cytoplasm into this swelling structure. Penetrating hyphae were also fluorescent. These observations suggested that transcriptional activation of *clpg2* occurred rapidly when the fungus entered its parasitic stage, implying that the hydrolytic enzyme encoded by this gene participated in host penetration.

Liu and Kolattukudy (1999) used GFP as a reporter to measure the expression of the calmodulin (*cam*) gene, one of the early genes expressed during fungal conidial differentiation of *M. grisea*. They fused the promoter *cam*_{Mg} to an EGFP segment cut out from pTEFEGFP (Vanden Wymelenberg *et al.*, 1997), and incorporated the construct into the *M. grisea* genome. GFP fluorescence was used as a measure of *cam* gene expression. Quantization of the fluorescence intensity of each cell was done by measuring the histogram of a rectangle surrounding each cell using confocal microscopy.

Siedenberg *et al.* (1999) tried to transform *Aspergillus niger* using wild type *gfp* and *sGFP* (S65T). *sGFP* (S65T) expression resulted in the formation of a functional fluorescent protein, while the expression of wild type *gfp* was not successful. Production of this functional fluorescent protein was found to be controlled by *A. niger* glucoamylase (*glaA*) gene promoter during the fungal growth under defined culture conditions. Similar GFP transformation research of *A. niger* was also done by Santerre Henriksen *et al.* (1999) to study *glaA* promoter.

A red-shifted variant, pEGFP-N1 was used as a reporter to monitor gene expression and food colonization by *Aspergillus flavus* (Du *et al.*, 1999). Fluorescence could be detected in cultures as young as 3 days old and persisted for more than 12 weeks in cultures maintained at room temperature. The intensity of fluorescence was sufficient to allow the visualization of a GFP-containing strain under a standard laboratory UV light. No special filters or equipment were needed to detect a GFP-expressing strain in culture or to monitor the colonization of corn

seeds with the strain. Bright fluorescence of the two transformants was most intense in conidia.

Rohel *et al.* (2001) studied infection of wheat and carbohydrate metabolism by *Mycosphaerella graminicola* using GFP fusion. *M. graminicola* was transformed with either a carbon source-repressed promoter – *sGFP* (Sheen *et al.*, 1995) fusion or a constitutive promoter – *sGFP* fusing construct. Monitoring the GFP level during fungal infection of wheat leaves revealed that the carbon source repression occurred after penetration until sporulation, when newly differentiated pycnidiospores fluoresced. GFP transformants allowed clear visualization of *M. graminicola* pathogenesis. Maximum fluorescence intensity was reached in penetrating structures.

Sexton and Howlett (2001) transformed *Leptosphaeria maculans*, a filamentous ascomycete that causes blackleg disease of oilseed *Brassica* spp. Two vectors were constructed: one for constitutive expression of GFP containing *egfp* and an *A. nidulans* promoter, and one for regulated expression of GFP containing *egfp* and a promoter of *L. mauculans* cyanide hydratase (*cht*) gene. Disease progression was followed, and gene expression during infection was studied. Very bright fluorescence observed in single plant cells and in cotyledon lesions may be associated with the formation of pycnidial initials. Cyanide hydratase was found to be transcribed in stem lesions. The strong fluorescence in cotyledon and stem lesions suggested that the fungus may produce the enzyme in areas where a high

degree of tissue damage is occurring, which may be due to the release of compounds such as nitriles during breakdown of plant cell compartments.

Ma *et al.* (2001) transformed a filamentous wood fungus, *Phanerochaete chrysosporium* with a GFP system containing the *egfp* and driven by the glyceraldehydes-*p*-dehydrogenase (*gpd*) or the manganese peroxidase (*mnp*) gene promoter from *P. chrysosporium*. GFP production under the control of the *gdp* promoter was observed under primary metabolic conditions, while GFP production under the control of the *mnp* promoter only occurred during secondary metabolic growth triggered by nutrient nitrogen source limitation and only under Mn²⁺ supplementation. Although transformed for reporting the gene expression, the transformant may also serve as a biomarker for viewing *P. chrysosporium* in wood or in a consortium of other fungi because the green fluorescence of GFP was located within the mycelium and was probably cytoplasmic. Fluorescence was spotty in some hyphal fragments, probably due to the formation of vacuoles in aging hyphae.

DeZwann *et al.* (1999) identified a novel plasma membrane protein that mediated appressorium differentiation of *M. grisea* in response to inductive substrate cues. The localization of this protein in the membrane was determined using GFP tagging.

Bowyer *et al.* (2000) used GFP fusion to monitor carbon metabolism of a plant pathogen *Tapesia yallundae* during infection of wheat. Stephenson *et al.* (2000) used GFP-promoter fusion proteins to monitor expression of a pathogenic

gene of *C. gloeosporioides* during infection of host *Stylosanthes guianensis*. Strong expression of GFP was observed in primary infection vesicles and in infected epidermal cells, while weaker expression was evident in hyphae growing within infected leaf tissue, suggesting this gene encodes a pathogenicity determinant associated with the biotrophic phase of primary infection.

Rojas *et al.* (2002) used GFP fusion to study a gene that encodes a member of a class of adhesions in a necrogenic bacterium *Erwinia chrysanthemi* that cause soft-rot diseases in plants. Mutants that lack this gene were less able to attach, aggregate, and consequently cause an aggregate-associated killing of epidermal cells, suggesting that bacterial attachment to host tissues was very important for its pathogenicity.

It is clear that there are numerous applications for GFP in fungal/plant interactions. While fungal/wood interactions have been examined, the field has received little attention. In this study, we will first transform a wood sapstain fungus, *Ophiostoma piceae* (Münch) Syd. & P. Syd., and a wood-inhabiting mold fungus, *Trichoderma harzianum* Rifai with a *gfp* gene, and then describe the applications of GFP as a biomarker to monitor the interactions between these two fungi in Douglas-fir wood.

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Chapter 2: Production of Protoplasts from Cultures of *Ophiostoma piceae*

Ying Xiao and Jeffrey J. Morrell

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Kyoto University
Uji, Kyoto 611-0011, Japan
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Production of Protoplasts from Cultures of *Ophiostoma piceae*

Ying Xiao and Jeffrey J. Morrell

Dept. of Wood and Science Engineering
Oregon State University, Corvallis, OR 97330, U. S. A.

ABSTRACT

A method is described for producing viable protoplasts from germinating conidia (germlings) of *Ophiostoma piceae*. The use of MgSO_4 -based osmotic stabilizers significantly improved protoplast release, as did the use of 20-hour-old germlings. Protoplast release rates also increased with higher enzyme concentrations and incubation times, but there were associated negative effects, including reduced regeneration. The results indicate that the use of young germlings, low lytic enzyme levels and short exposure periods produced the most viable protoplasts.

Key words: *Ophiostoma piceae*, bluestain, protoplasts, biological control

INTRODUCTION

Discoloration of lumber by fungi is a major cause of economic loss for wood processors. From the time it is cut in the forest until it dries to below 20% to 25% moisture content or later, if it is inadvertently rewetted, wood can be rapidly colonized by a diverse array of fungi that can discolor the surface via the production of pigmented spores or the interior through melanized hyphae in the cell lumens. Although the precise costs that result from fungal discoloration are difficult to determine, it has been estimated that they cause approximately 10 million dollars in losses/year (Scheffer, 1973). Surveys of lumber producers in the western United States suggest that the figure is extremely conservative (Anderson *et al.*, 2002). Among the most important fungi causing discoloration of lumber are the *Ophiostoma* species. Many of these species are widely distributed and cause deep discoloration of freshly sawn wood. *Ophiostoma piceae* is an economically important fungus in many coniferous species in western North America.

Discoloration of lumber has generally been controlled by drying the wood below 20% or, where that was not feasible, applying surface coatings of prophylactic fungicides. Most mills lack the kiln capacity to dry all their production, however, and there are some applications where the user does not want dry wood. Chemicals carry with them the risk of worker exposure and the potential for scrutiny by an increasingly chemophobic public.

One alternative to chemical treatment is biological control (biocontrol): using one organism to control the detrimental activities of another. Biocontrol is

not a new concept; fungi have been employed for this purpose on a variety of wood-based materials (Rishbeth, 1963; Ricard, 1976; Bruce and King, 1986), but there have been few broadly successful applications against stain and mold fungi (Freitag *et al.*, 1992; Behrendt *et al.*, 1995; Kay, 1997). Failures to achieve successful biocontrol have been attributed to the inability of the biocontrol organism to function under field conditions, where growth is limited by competing organisms and environmental conditions may be less ideal. The failures have highlighted the need for more fundamental research on *in situ* microbial interactions between biocontrol agents and their intended targets.

An important aspect of this need is the ability to study microbial interactions within wood. This is generally difficult when the biocontrol agent is a fungus, since most of the hyphae of both the stain fungi and the biocontrol agent are hyaline, making it difficult to determine which fungi are present. A number of fluorescent probes are available that are specific for components in fungal hyphae, but most of these react with hyphal components common to many fungi (Morrell *et al.*, 1985; Xiao *et al.*, 2000).

One approach to this dilemma is to transform one of the test fungi by inserting genetic material capable of producing fluorescent proteins that are continuously expressed. This process would allow visualization of all of the hyphae of that species in wood using fluorescence microscopy. An important first step in this transformation is to develop systems to get the genetic material into the cells. The simplest method for accomplishing this task is to generate fungal

protoplasts. Protoplasts are the wall-less states of cells after the cell walls have been removed through enzymatic digestion. Many factors including type and concentration of enzymes, treating time, type of osmotic stabilizers, fungal species, and culture age can have pronounced effects on the yield of protoplasts and their regeneration (Davis, 1985). Protocols for protoplasting have been reported for some wood inhabiting fungi (Gold *et al.*, 1983; Kitamoto *et al.*, 1984; Nutsubidze *et al.*, 1990; Trojanowski and Huttermann, 1990; Royer *et al.*, 1991; Chen and Morrell, 1993; Richards, 1994; Bartholomew *et al.*, 2001). The conditions for protoplasting varied widely in these studies, suggesting the need to define optimal conditions for protoplasting for each species.

There are no reports of procedures for generating protoplasts from *O. piceae*. In this report, we outline procedures for producing protoplasts of *O. piceae* as a prelude to transformation.

MATERIALS AND METHODS

Cultures of *O. piceae* (Isolate AU135-1, Forintek, Canada Corp.) were maintained on 1% malt extract agar. The strain was originally isolated from white spruce lumber in British Columbia, Canada. Prior to use, the fungus was inoculated on an agar media containing 1.0% malt extract, 1.0% glucose, 0.1% yeast extract, and 1.5% agar (MGY agar), and incubated for 12 days at 25 °C in darkness. The test fungus produced abundant conidia on this medium in

preliminary tests. Conidia forming on the agar surface were gently washed from the plates using sterile distilled water. Conidia were used either directly for protoplasting or were used to produce germlings. Conidia for direct use were collected by centrifugation ($7500 \times g$ for 5 min). Conidia were washed two times with distilled water, then one time with one of two osmotic stabilizers, either 0.5 M MgSO_4 or 0.5 M mannitol in 50 mM maleic-NaOH (pH 5.5) (Nutsubidze *et al.*, 1990). Conidia were then transferred to a 16- x 300-mm-long sterile glass tube.

Germlings were produced by adding approximately 1×10^8 conidia into 100 mL of a media containing 2% potato dextrose and 0.1% malt extract (PDB Broth). The broth was incubated for 16, 20, or 24 hours on a rotary shaker (100 rpm) at room temperature ($20\sim 23^\circ \text{C}$) to produce germlings. Germlings were collected by filtration through a 20 μm nylon mesh (Sefar America, Depew, NY). The germlings were washed three times with sterile distilled water, then three times with osmotic stabilizers, either 0.5 M MgSO_4 or 0.5 M mannitol in 50 mM maleic-NaOH (pH 5.5). Wet germlings were then transferred to a 16- x 300-mm-long sterile glass tube.

Protoplast Production

Lyophilized lysing enzyme produced by *Trichoderma harzianum* (Sigma Chemicals, St. Louis, MO) was dissolved in the appropriate osmotic stabilizer at 0.4, 0.7, or 1.0% (wt/wt). The same osmotic stabilizer was used throughout all

procedures for a single treatment. The resulting solution was sterilized by filtration through a 0.22 μm membrane.

Ten mL of lysing enzyme was added to test tubes containing conidia or germlings. The tubes were incubated for 30, 60, or 120 minutes on a nutator (Clay Adams, Sparks, MD) at 25 °C. The solution was diluted with the appropriate osmotic stabilizer to 20~25 mL, and filtered through 10 μm nylon mesh. The filtered solution was centrifuged at $7500 \times g$ for 10 minutes and the supernatant was decanted and discarded. The pellet was resuspended in 20 mL of the appropriate osmotic stabilizer and centrifuged again. After resuspension, protoplasts and hyphal fragments were counted with a hemacytometer.

There were three replicates for each trial. For each replicate, one ml of protoplast solution containing approximately 1×10^7 cells received 100 μL of a solution containing 50 $\mu\text{g/mL}$ of fluorescent isothiocyanate (FITC) coupled wheat-germ agglutinin (Morrell *et al.*, 1985; Xiao *et al.*, 2000). The solution was incubated in darkness for 30 minutes, then cells were collected by centrifugation ($2500 \times g$ for 5 min), rinsed with 1 mL of the appropriate osmotic stabilizer, and centrifuged. The resulting cells were observed with a Nikon Elipse E400 microscope equipped with a filter specific for FITC, and the numbers of fluorescent and total protoplasts were counted.

Protoplasts were produced from conidia by adding 10 mL of lysing enzyme solution to washed conidia in the appropriate osmotic stabilizers. These tubes were incubated on the nutator shaker for 2, 4, or 24 hours at room temperature. Each

variable was investigated on three replicates. Protoplast production was assessed as described above.

Protoplast Regeneration

Although it is important to be able to remove cell walls, fungal protoplasts can only remain in this state for relatively short periods and must eventually be allowed to regenerate cell walls. The use of harsh conditions can adversely affect the ability of the protoplast to regenerate. Protoplast regeneration was assessed by adding 1 mL of protoplasts that had previously been diluted to 1×10^4 protoplasts/mL in the appropriate osmotic buffer into 9 mL of molten MGY medium containing 1.2M sorbitol. This mixture was placed in a plastic petri dish and incubated for four days at room temperature. Regeneration was then assessed by counting the colonies. The percentage of the colony-forming units produced from the total cells in the dilution was defined as the regeneration rate.

Data Analysis

The effects of enzyme concentration, osmotic stabilizer, and treatment time were assessed using Multiple Linear Regression, and treatment measures were compared using Fisher's Least Significant Different test on SAS (SAS ver 7.0, SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Protoplasts could not be produced from conidia, even when conidia were exposed to the lytic enzyme for 24 hours. This suggests that cell wall components in the conidia were less susceptible to the chitinase systems produced by *T. harzianum*. As a result, all data reported are for germlings.

Effects of Buffer on Protoplast Production

Protoplast levels were significantly greater ($p\text{-value} < 0.001$) when MgSO_4 was incorporated into the osmotic stabilizer. Average protoplast production ranged from 0.79 to 3.15×10^7 protoplast/mL when mannitol was used, compared with 22.13 to 28.48×10^7 protoplasts/mL in MgSO_4 (Table 2.1).

Table 2.1: Number of protoplasts produced from germlings exposed to 0.4% to 1.0% lytic enzyme in MgSO_4 -maleic-NaOH or mannitol -maleic-NaOH stabilizer for 1 hour

Stabilizer	Protoplasts ($\times 10^7$ /mL)		
	Enzyme concentration (%)		
	0.4	0.7	1.0
MgSO_4 -maleic-NaOH	22.13 (0.46) ¹	27.77 (12.49)	28.48 (2.12)
Mannitol-maleic-NaOH	0.79 (0.12)	1.55 (0.09)	3.15 (0.39)

¹ Values represent means of 3 replicates with the standard deviations in the parenthesis.

Protoplast levels also tended to increase steadily with increasing enzyme concentration, suggesting that the high concentrations of lytic enzymes enhanced cell wall removal (Table 2.1).

These preliminary trials indicated that the MgSO_4 -based osmotic stabilizer produced more protoplasts than did mannitol; this stabilizer was therefore used in all subsequent tests.

Effects of Germling Age on Protoplast Production

Wet mycelial mass tended to increase with incubation period (Figure 2.1); however, protoplast production peaked after 20 hours of incubation and was significantly higher at that point than after 16 or 24 hours ($p < 0.001$) (Figure 2.2). These results suggest that early stages of mycelial growth may be most amenable to protoplasting. Hyphal thickening and senescing away from the growing tip may reduce the potential for protoplast production. Conversely, the presence of hyphal fragments tended to steadily decline as germling age increased, although the differences were not significant. The presence of higher levels of hyphal fragments in the germling suspension is undesirable because they consume enzyme and suggest the presence of non-viable hyphal fragments that could not release protoplasts. Incubation for 20 or 24 hours appeared to markedly reduce the levels of hyphal fragments.

Enzyme concentration and treatment time both significantly affected protoplast release ($p < 0.0001$ and $p = 0.0002$, respectively; Table 2.2). Mean

number of protoplasts produced was predicted to increase by 1.857×10^8 protoplasts per mL for every 0.3% increase in enzyme concentration at a given incubation time, and by 0.95×10^8 protoplasts/mL for every 30 minutes of incubation at a given enzyme level. These results are not surprising since cell wall removal depends on both enzyme concentration and exposure time (Davis, 1985).

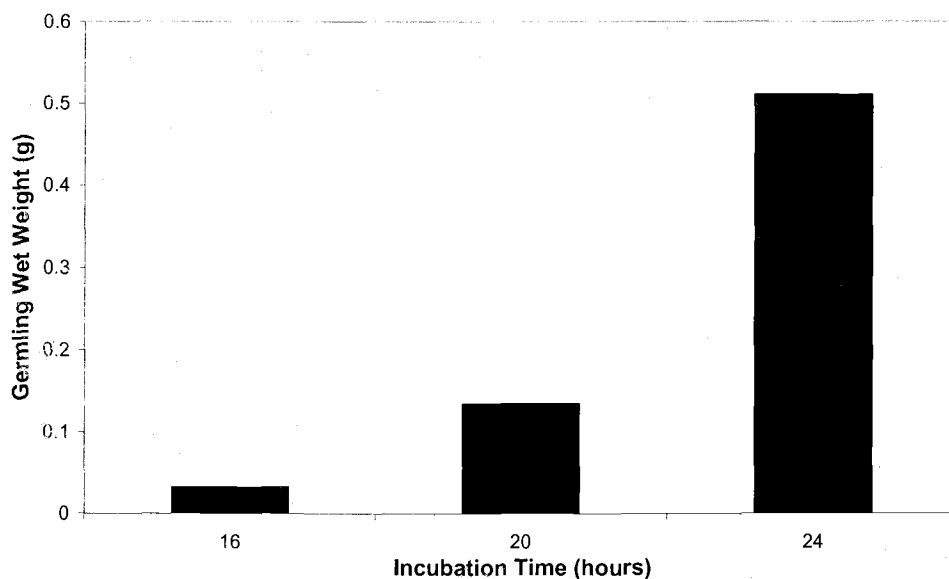


Figure 2.1: Effect of incubation time on wet weight of germlings of *Ophiostoma piceae* in MgSO_4 -maleic-NaOH buffer with 1.0% lytic enzyme.

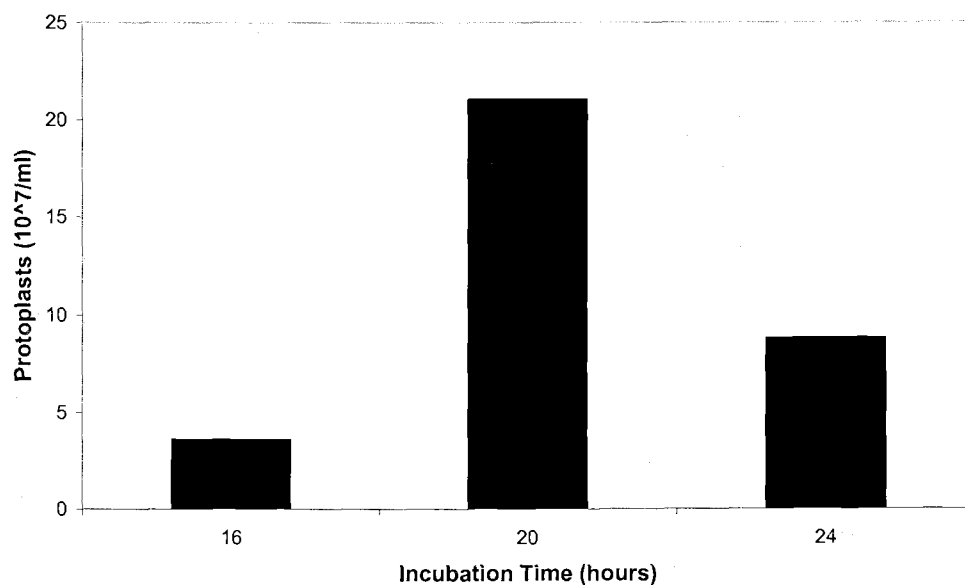


Figure 2.2: Effect of incubation time on protoplast production from germlings of *Ophiostoma piceae* in MgSO_4 -maleic-NaOH buffer with 1.0% lytic enzyme.

Increasing enzyme concentrations and treatment times were also both associated with higher percentages of hyphal fragments, which consume enzyme, but do not contribute to protoplast production ($p < 0.0001$ and $p = 0.0019$, respectively). It is unclear why these fragments increased in frequency. Older hyphae might have been fragmented due to incomplete cell wall lysis but failed to release protoplasts.

Table 2.2 Effect of enzyme concentration and exposure period on release of protoplasts from 20-hour-old germlings and subsequent regeneration of hyphae from these treatments

Enzyme concentration (%)	Incubation period (min)	Protoplasts ($\times 10^7$ /mL)	Protoplasts with cell wall (%)	Hyphal fragments (%)	Regeneration (%)
0.4	30	3.32 (1.14) ¹	3.74 (0.31)	6.84 (1.98)	— ²
	60	22.13 (0.46)	13.99 (6.73)	16.73 (6.46)	1.35 (0.18)
	120	30.75 (2.08)	10.62 (0.54)	19.79 (5.76)	0.68 (0.01)
0.7	30	29.07 (4.08)	3.75 (0.81)	15.73 (3.15)	2.17 (0.33)
	60	27.77 (12.49)	4.44 (0.46)	22.74 (8.09)	0.64 (0.13)
	120	23.37 (5.64)	14.48 (1.39)	21.84 (2.53)	1.61 (0.13)
1.0	30	17.28 (2.11)	5.22 (1.28)	29.39 (1.59)	1.07 (0.10)
	60	28.48 (2.12)	7.98 (1.29)	23.59 (1.54)	1.12 (0.27)
	120	37.63 (7.46)	8.49 (2.32)	42.36 (6.42)	0.42 (0.07)

¹ Values represent means of 3 replicates with the standard deviations in the parenthesis

² There were too few protoplasts to evaluate regeneration.

Cells that fluoresce after exposure to FITC-WGA are indicative of incomplete cell wall removal on the protoplast because this stain is specific for chitin. In theory, the frequency of fluorescing cells should decline with increasing enzyme concentration or treatment time. Increasing enzyme concentration for a given treatment time did not significantly reduce the percentage of fluorescing cells, while increasing treatment time significantly increased the percentage of

fluorescing protoplasts ($p < 0.0001$). These results imply that there was an excess of enzyme available for reaction, but that other factors, such as site access, affected cell wall removal. As a result, increasing enzyme levels had no effect on the residual cell wall. Increasing the treatment time should allow more reactions to occur and thereby improve cell wall lysis, but longer exposures also will allow lysis of cell walls on older hyphae. The cell wall polymers on these hyphae are likely to be more heavily cross-linked and therefore less likely to be completely removed (Wessels, 1990).

Protoplast Regeneration

Although protoplast production is important, it is equally critical that the conditions used to produce protoplasts do not damage cell functions to the point where regeneration of fungal hyphae is precluded. Excessive enzyme concentrations, prolonged treatment times, and poor buffer choices are among the factors that can hinder regeneration.

Regeneration rates of *O. piceae* were generally low, ranging from 0.4% to 2.53% for the various treatments. Increasing enzyme concentration from 0.4% to 0.7% at a given treatment time had no significant effect on regeneration, while increasing the concentration to 1.0% had a significant negative effect. Similarly, increasing treatment time to 120 minutes had a significant negative effect on regeneration. These results indicate caution should be exercised to ensure that

enzyme levels and exposure times adjusted to enhance protoplast release do not produce long-term impacts on cell viability and function.

Protoplasting from conidia of *O. piceae* was not successful in this study. Pretreatment of conidia in 2-mercaptoethanol or dithiothreitol followed by prolonged incubation in enzyme solution may be useful, but the treatments must be long (Royer *et al.*, 1991; Richards, 1994). Protoplasts were easily produced from 20-hour-old *O. piceae* germlings after 30 minutes of incubation. Clearly, protoplasts can be produced from *O. piceae* germlings with relative ease because this incubation time was short compared to those reported in other studies (Gold *et al.*, 1983; Kitamoto *et al.*, 1984; Royer *et al.*, 1991; Chen and Morrell, 1993; Richards, 1994). However, regeneration rates were relatively low compared to other studies (Kitamoto *et al.*, 1984; Chen and Morrell, 1993; Richards, 1994). Most protoplasts appeared to be intact under a light microscope, suggesting that low regeneration rates may be due to poor regeneration conditions. Further studies will be required to more completely define optimum conditions for both protoplast release and regeneration.

CONCLUSIONS

Protoplasts could be produced from 20-hour-old germlings of *O. piceae* using a MgSO_4 -maleic-NaOH buffer. Higher enzyme concentrations and treatment times increased protoplast release but reduced the ability of the protoplasts to

regenerate. Exposure to 0.7% lytic enzyme for 30 minutes appeared to produce optimum release and regeneration for the conditions evaluated.

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Chapter 3: Transformation of *Ophiostoma piceae* and *Trichoderma harzianum* with a green fluorescent protein (GFP) gene

Ying Xiao, Lynda M. Ciuffetti and Jeffrey J. Morrell

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Transformation of *Ophiostoma piceae* and *Trichoderma harzianum* with a green fluorescent protein (GFP) gene

Ying Xiao¹, Lynda M. Ciuffetti² and Jeffrey J. Morrell¹

¹ Dept. of Wood and Science Engineering, ² Dept. of Botany and Plant Pathology

Oregon State University, Corvallis, OR 97330, U.S.A.

ABSTRACT

While microbial colonization of wood is presumed to be characterized by a myriad of interactions between numerous organisms, studying these processes is often difficult owing to the opaque nature of the wood and the inability to readily distinguish among the many species colonizing the material. One method for enhancing the ability to distinguish organisms is to induce specific proteins in one or more organisms that can be detected using fluorescence or other light microscopic techniques. The insertion of genes for the production of green fluorescent proteins produced by the jellyfish, *Aequora victoria*, has been widely used to visualize a variety of organisms. In this report, we describe transformation of two fungi, *Ophiostoma piceae* and *Trichoderma harzianum* using a green fluorescent protein (SGFP) gene under the control of the *ToxA* promoter of *Pyrenophora tritici-repentis*. The growth and wood colonization by two transformed fungi were compared to their non-transformed strains. The expression of *gfp* was particularly useful for studying the spatial distribution of young hyphae in wood.

Key Words: green fluorescent protein (GFP) transformation, *Ophiostoma piceae*, *Trichoderma harzianum*, wood.

INTRODUCTION

Wood is an organic material that can be colonized by a complex matrix of microorganisms. Understanding fungi that colonize wood is critical for developing effective methods for wood protection; however, the opaque nature of wood makes it difficult to study fungal growth *in situ*. In addition, it is also difficult to use ordinary microscopy to distinguish hyphae of a specific fungus from others due to the similarity in fungal morphologies. Immuno-labelling has the potential for detecting specific fungi in wood, but its application is limited by non-specific wood binding (Xiao *et al.*, 1999). Fungal hyphae can also be visualized using fluorescent coupled lectins, but these materials do not allow individual species to be differentiated (Morrell *et al.*, 1985; Xiao *et al.*, 2000). The green fluorescent protein (GFP) technique has been widely applied for visualizing living cells (Valdivia *et al.*, 1996; Bae and Knudsen 2000; Haraguchi *et al.* 2000; Lorang *et al.*, 2001; So *et al.*, 2002). A gene isolated from the jellyfish, *Aequora victoria*, is inserted into the target fungus, resulting in production of a fluorescent strain of the fungus. This technique enables the visualization of the behavior of the transformed fungus using fluorescence microscopy.

Expression of *gfp* in filamentous fungi requires a *gfp* variant that is efficiently translated in fungi, a transformation system, and a fungal promoter that satisfies the requirements of a given experimental objective (Lorang *et al.*, 2001). In contrast to studies on *gfp* transformation of microorganisms of interest in the medical and agricultural fields, there is limited information on *gfp* transformation of wood inhabiting fungi (Ma *et al.*, 2001; Lee *et al.*, 2002). A sapstain fungus, *Ophiostoma piceae* (Münch) Syd. and P. Syd., was transformed using the *gGFP* vector (Maor *et al.*, 1998) containing the *SGFP* and *hph* (hygromycin B phosphotransferase) genes under the control of *Aspergillus nidulans gpd* promoter (Lee *et al.*, 2002). The authors concluded that GFP was a useful biomarker for *O. piceae* since the GFP-expressing fungus showed normal phenotypes, growth and colonization on wood. However, we were not aware of this parallel study until after we successfully transformed the same fungus but a different strain. Another fungus that is of interest to us is a potential biocontrol agent against *O. piceae* in wood, *Trichoderma harzianum* Rifai. A strain of *T. harzianum* isolated from soil was cotransformed with β -glucuronidase and green fluorescent protein genes previously (Bae and Knudsen, 2000). A plasmid pTEFEGFP vector containing *EGFP*, the *Aureobasidium pullulans* translation elongation factor promoter was used. This *gfp* transformed *T. harzianum* strain may not be a proper choice for our study because of its isolation origin (soil, not wood).

A fungal transformation vector, pCT74, that expresses *SGFP* under the control of the *ToxA* gene promoter (Ciuffetti *et al.*, 1997) from *Pyrenophora*

tritici-repentis has proven useful for expressing *gfp* in a number of fungi that belong to the Ascomycota (Lorang *et al.*, 2001). This vector may be useful for transforming sapstain fungi, an important group of wood inhabiting fungi, most of which belong to the Ascomycota. In this paper, we report the transformation of *O. piceae* and *T. harzianum* using pCT74. The growth and wood colonization of the two transformed fungi were compared to their non-transformed strains.

MATERIALS AND METHODS

O. piceae isolate AU135-1 isolated from white spruce was kindly provided by Dr. Adnan Uzunovic at Forintek, Canada Corp (Vancouver, B. C. Canada). *T. harzianum* Rifai isolate 15B isolated from Douglas-fir roots was supplied by the U.S.D.A. Forestry Science Laboratory in Corvallis, Oregon. The vector, pCT74, was developed in Dr. Lynda Ciuffetti's laboratory (Dept. of Botany and Plant Pathology, Oregon State University) (Lorang *et al.*, 2001). All chemicals and media materials were purchased from Sigma Chemicals (St. Louis, MO) or Fisher Scientific (Tustin, CA) unless otherwise specified.

Protoplasting and *gfp* Transformation

O. piceae: The protoplast procedures were optimized prior to transformation to maximize production and viability (Xiao and Morrell, in press).

Briefly, 20-hr old germlings (wet weight: 0.7 - 0.8 g) of the test fungus were treated with 0.7% (wt/wt) lysing enzyme in 0.5 M MgSO_4 -Maleic-NaOH buffer (pH=7.4) for 30 minutes. Protoplasts were collected and washed by centrifugation, and then resuspended in 1.2 M STC (1.2 M sorbitol, 10mM Tris-HCL, pH 7.5, and 10 mM CaCl_2) (Vollmer and Yanofsky, 1986) to a final concentration of 10^8 protoplasts per mL and kept on ice until needed.

Fungal transformation was based on the procedures described by Ciuffetti *et al.* (1997). Spermidine (2 μL of 50 mM), plasmid pCT74 (10 μg), and 2×10^7 protoplasts in 200 μL of STC were gently mixed in a test tube, and incubated at room temperature for 15 min. Three hundred μL of polyethylene glycol 3350 (PEG 3350) (40% in 50 mM Tris, pH 8.0, and 50 mM CaCl_2) was added slowly and mixed by gently rolling the tube. Another 1 mL of 40% PEG was then added to the tube. Nine milliliters of molten regeneration medium (RM) (0.6 M sucrose, 2% potato dextrose agar (PDA)) was added to the mixture after 20 min of incubation and poured onto solidified RM supplemented with 50 μg of hygromycin B to give a final concentration of 25 $\mu\text{g}/\text{mL}$. Protoplasts were plated at 4×10^6 protoplasts per plate. Plates were incubated in the dark at 25°C, and observed using a Leica MZFLIII stereo-fluorescence microscope (excitation: 450 - 490 nm, emission: 500 - 550 nm). Hygromycin-resistant transformants were evident after 1 day of incubation. Subculturing was carried out after 10 days of incubation.

T. harzianum: The protoplast procedures were optimized prior to transformation to maximize production and viability for transformation. Briefly, 21-hr old germlings were reacted with 1% (wt/wt) driselase and 1% (wt/wt) glucuronidase in 0.5 M MgSO₄-Maleic-NaOH buffer (pH=7.4) for 1 hr. The transformation procedures were the same as those described for *O. piceae* except that the solidified RM was supplemented with 100 µg of hygromycin B to give a final concentration of 50 µg/mL. The hygromycin-resistant transformants were evident after 3 or 4 days of incubation. The transformants were subcultured at various time intervals (7-30 days) following the initial incubation.

Wood Test

Transformants were grown on PDA or in wood blocks, and growth was compared with the non-transformed strain of each fungus. Freshly sawn Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) sapwood lumber was cut into wood blocks (10 × 20 × 40 mm) and sterilized by exposure to 2.5 Mrads of ionizing radiation from a cobalt 60 source. The wood blocks were dipped for 15 - 20 seconds into spore suspensions of each fungus at a concentration of 1×10^7 spores per mL. Excessive solution was absorbed from a corner of each sample using a sterile cotton swab. The inoculated blocks were then put onto glass rods in glass petri dishes containing moistened filter paper on the bottom. Moisture in the petri

dish was replenished periodically. All blocks were incubated at room temperature (20 - 23 °C) in the dark. There were six replicates for each treatment.

Wood surfaces were observed using a Nikon Elipse E400 microscope equipped with a green fluorescence filter set (excitation: 465 - 495 nm, emission: 515 - 555 nm) and a red fluorescence filter set (excitation: 515 - 565nm, emission: 600 - 660 nm). Paint Shop Pro (Ver 5.0) (Jasc Software Inc., Eden Prairie, USA) was used to overlay images obtained through the green and red filters respectively. This overlay allowed us to determine which hyphae fluoresced at a given wavelength. Two destructive assessments were carried out after 4 and 10 weeks of incubation to assess the internal degree of staining. A wood block was cut in half at the middle of the radial face to expose the middle tangential face. One half of each sample was then cut in the middle of the tangential face to expose the middle radial face (Figure 3.1). The degree of discoloration was estimated on a 0 (no discoloration) to 100 (complete discoloration) scale. Radial sections (30 – 50 µm thick) were cut using a microtome, then the surfaces of the smoothed middle radial faces were directly examined using fluorescence microscopy. Wood sections were mounted in water on glass slides directly or after staining with Rhodamine coupled wheat germ agglutinin (WGA) (Molecular Probes, Eugene, OR) (Xiao *et al.*, 2000). The percentage of wood cells colonized by hyphae in a given section was then estimated.

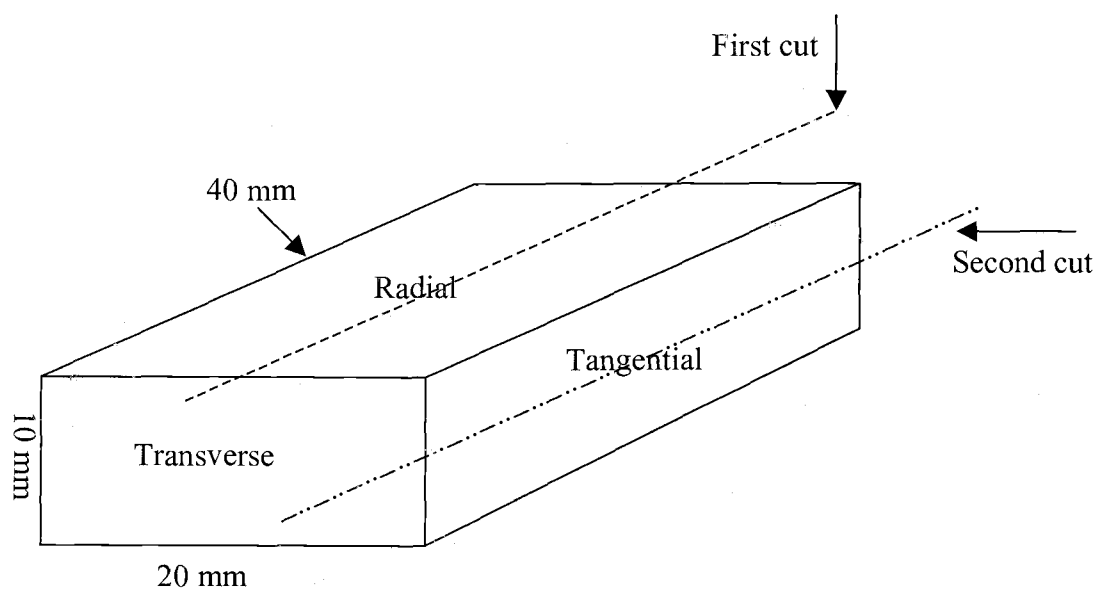


Figure 3.1: Patterns used to cut wood blocks for destructive assessments of internal fungal stain.

Data Analysis

Values for surface and internal discoloration produced by non-transformed and transformed *O. piceae* and *T. harzianum* were compared using Tukey's HSD multiple comparison method or a two-sample t-test on SAS (SAS ver 8.2, SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Transformation

O. piceae: Approximately 50 - 100 bright fluorescent colonies grew on each transformation plate, but the exact numbers were difficult to obtain due to overlapping colony growth. To avoid the overlap of transformed colonies, *O. piceae* protoplasts could be plated at a lower density. Ten of these colonies were subcultured and purified by cutting a single hyphal tip from the actively growing edge of a fungal colony. All purified strains showed similar fluorescence and colony growth morphology on hygromycin B selection medium. After purification, three transformants, OPGF-I, OPGF-II, and OPGF-III, were selected and subcultured ten successive times on PDA without selection pressure to test mitotic stability. All three strains were stable over the transfers, retaining the *gfp*, and pigmented similarly to the non-transformed strain on PDA.

T. harzianum: Fluorescent colonies were visualized after 3 days of incubation and rapidly expanded into irregular shapes with uneven fluorescence among the colonies. Six bright colonies emerged upon prolonged incubation. These six colonies were subcultured, purified, and tested for stability as described above for *O. piceae*. One stable transformant, THGF-I, was obtained.

Wood Test

Surface Observations

O. piceae: The three *O. piceae gfp* transformants showed germination rates on wood that were comparable to those found on artificial media. At 22 hrs, well-developed germ tubes were clearly visible (Figure 3.2a). The non-transformed *O. piceae* was not fluorescent and could not be observed using fluorescence microscopy at this stage (Figure 3.2b). It was also very difficult to observe non-transformed *O. piceae* on wood using ordinary stereo-light microscopy due to the hyaline nature of *O. piceae* propagules. Transformants expressing *gfp* transformation provided a convenient tool for monitoring fungal growth on wood at the early stages of colonization.

Numerous brightly-fluorescent synnemata emerged on the wood surface two days after inoculation, but these synnemata were still invisible to the naked eye (Figure 3.3). These brightly-fluorescent synnemata rapidly pigmented and started to become visible to the naked eye by the fourth day. Well-developed pigmented synnemata with spore heads covered all wood blocks after 4 days, representing the mature *Graphium* state of this fungus. Although it was difficult to observe the surface growth of the non-transformed *O. piceae* in the first two days, the visible development of black synnemata was comparable for the non-transformed strain and the *gfp* transformants.

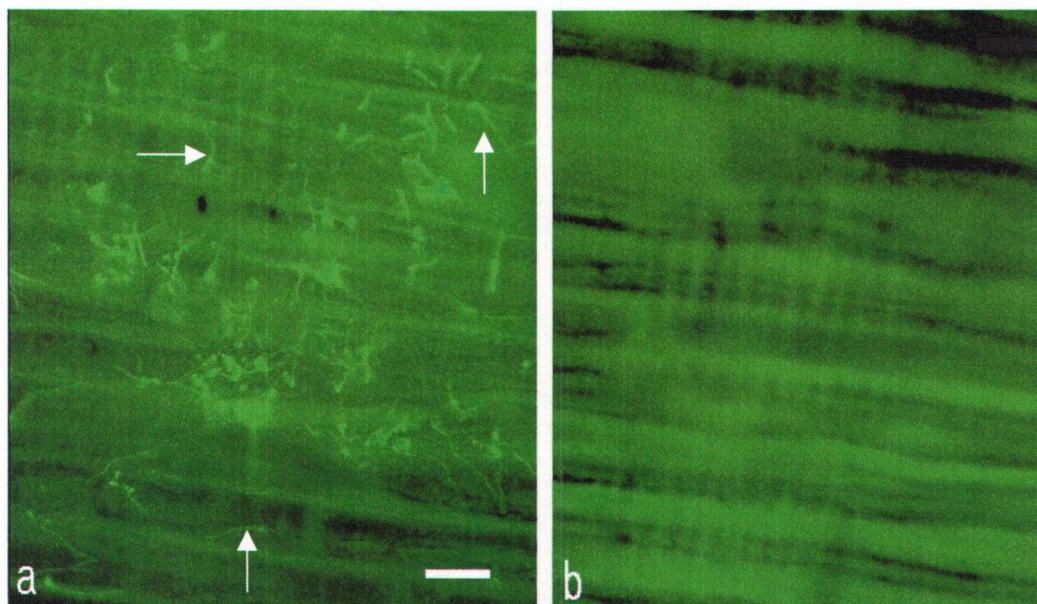


Figure 3.2: Fluorescence micrographs showing a) spore germination of *O. piceae* transformed with pCT74 at 22 hrs after inoculation on fresh Douglas-fir sapwood (arrows indicate germination tubes) or b) Douglas-fir sapwood inoculated with non-transformed *O. piceae* at 22 hrs after inoculation. Note the inability to resolve the germinating spores of the non-transformed *O. piceae* (Bar: 50 μ m).

***T. harzianum*:** The *T. harzianum* *gfp* transformant germinated more slowly than *O. piceae* on fresh Douglas-fir sapwood. *T. harzianum* was not observed on the wood surface until after spore germination, which may be the result of blocked fluorescence in the spores due to the green pigmentation. Spore germination was not observed in the first two days, suggesting that the spores might still be at the swollen stage. Growth was rapid once the spores germinated on the fourth day, and mycelia covered the wood blocks by the fifth day (Figure 3.4). After seven days of incubation, scattered light green spore masses were observed on both the non-transformant and transformant treated wood blocks. The areas covered by spore

masses appeared to stabilize around the tenth day, then spores developed a dark green color. Sporulation of the non-transformed and transformed *T. harzianum* were comparable.

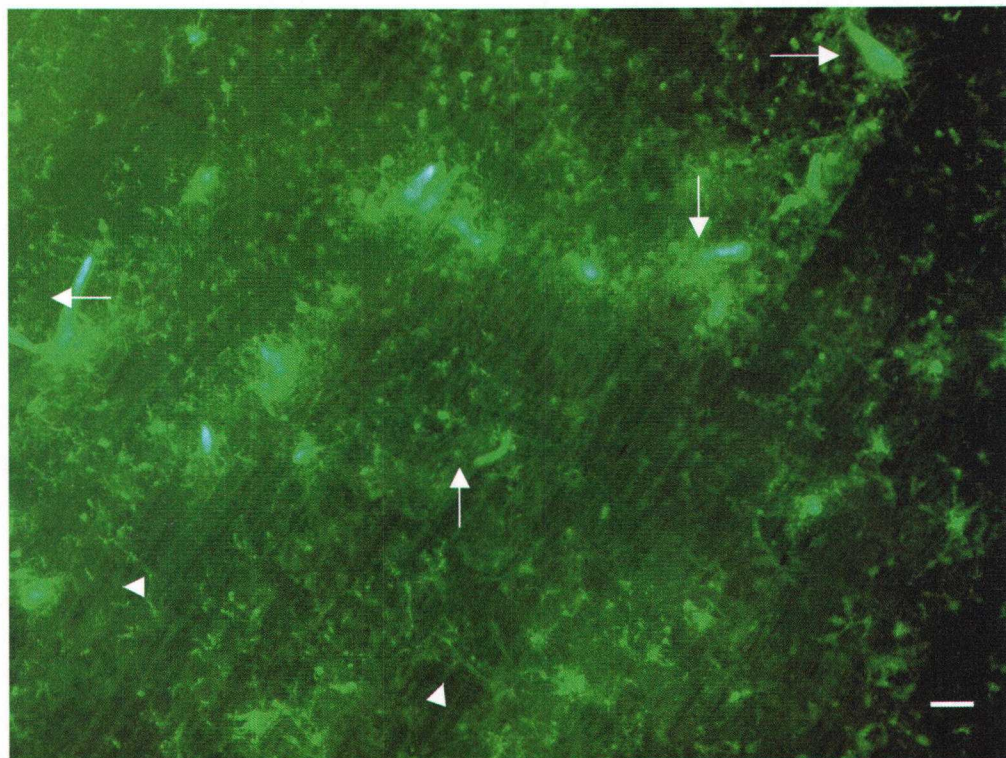


Figure 3.3: Fluorescence micrograph showing growth of *O. piceae* transformed with pCT74 at 48 hrs on fresh Douglas-fir sapwood. Arrows indicate synnemata before pigmentation and arrowheads indicate hyphae (Bar: 100 μ m).

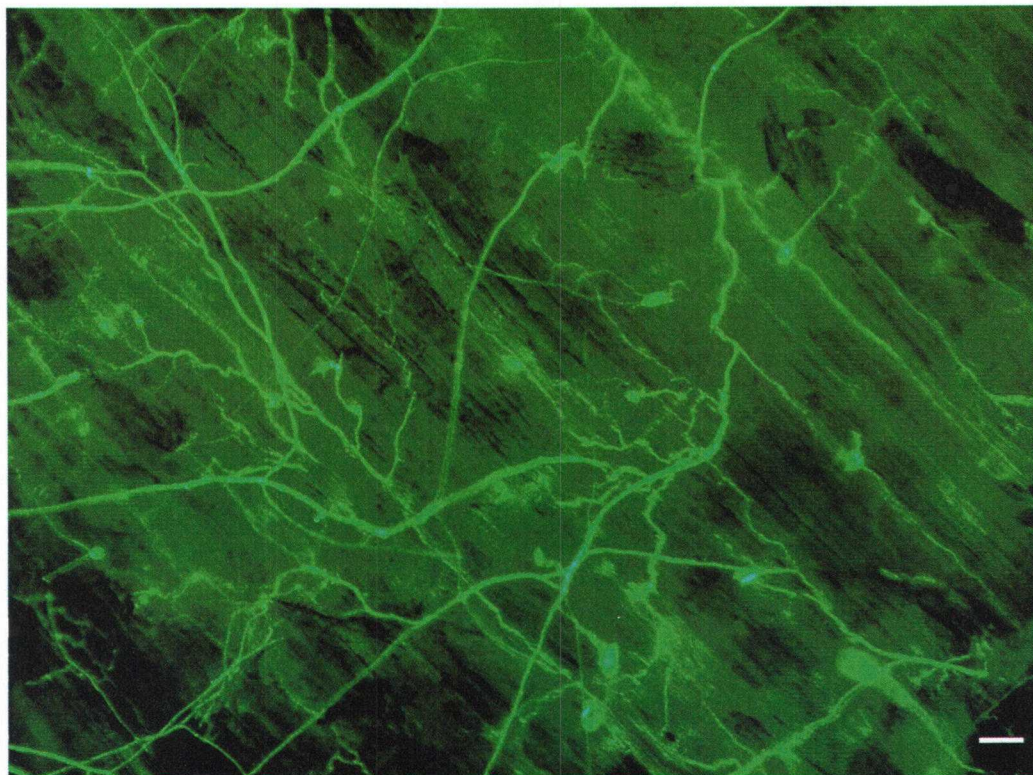


Figure 3.4: Fluorescence micrograph showing hyphal growth of the *T. harzianum gfp* transformant on fresh Douglas-fir sapwood after four days of incubation (Bar: 100 μ m).

Assessment

***O. piceae*:** Vigorously growing hyphae were brightly fluorescent with the GFP protein, while older cells were less fluorescent. WGA is a chitin specific fluorescent stain that is useful for localizing fungal hyphae in wood (Morrell *et al.*; 1985, Xiao *et al.*, 2000). However, WGA was found to be less reactive on vigorously growing hyphae in this study, suggesting that the chitin content was low in their cell walls (Figure 3.5a & b). The ability to visualize young hyphae in wood may be valuable for studying initial establishment of biocontrol agents. The

simultaneous use of WGA and GFP would allow researchers to distinguish between actively growing and mature hyphae in a wood section.

Hyphae of this *O. piceae* strain did not pigment heavily in Douglas-fir sapwood, resulting in a light brown color inside the wood. However, darkly pigmented hyphae were observed on the surface at the bases of black synnemata, and wood surfaces showed dark stained spots after the synnemata were removed. There was no significant difference in the ability of the non-transformed and *gfp* transformed strains to superficially or internally stain the wood (Table 3.1). These results indicate that transformation did not affect the fitness of the organism as a stain fungus.

Wood sections without WGA staining showed little evidence of fluorescing hyphae after 4 weeks, while numerous red fluorescing hyphae were observed on WGA stained wood sections. Fungal hyphae were abundant at the ends of wood sections. In contrast, hyphae were relatively sparse and randomly distributed over the central parts of wood sections.

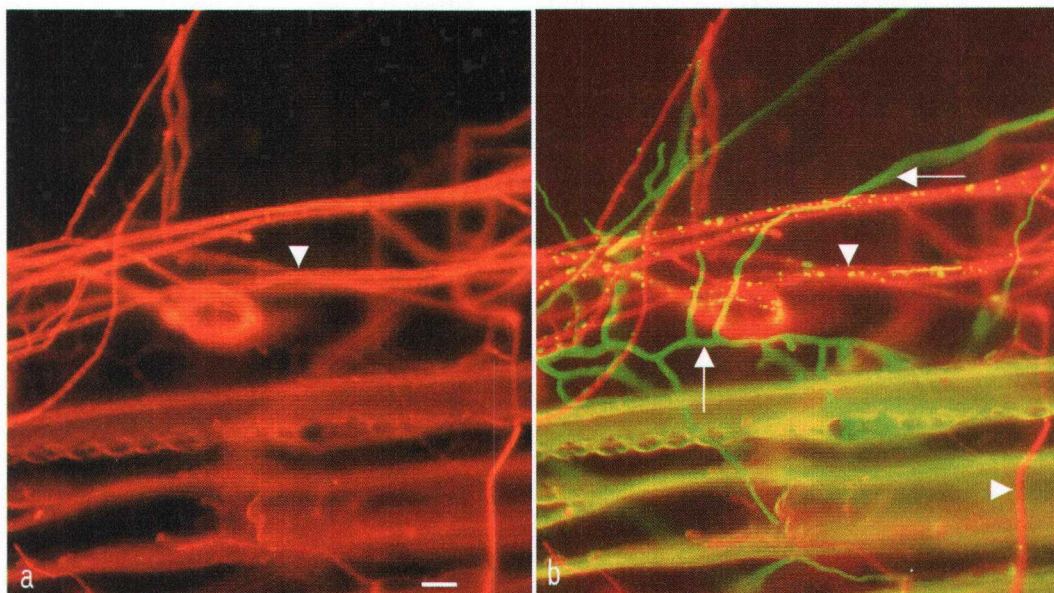


Figure 3.5: Fluorescence micrographs showing fungal hyphae stained a) with Rhodamine coupled WGA (arrowheads) or b) an overlay image of the same field with both Rhodamine coupled WGA (red filter) and GFP (green filter). Arrows show young hyphae expressing *gfp*. Arrowheads: hyphae stained with Rhodamine coupled WGA (Bar: 20 μ m).

There was little difference in both superficial and internal stain between the four and ten week assessments (Table 3.1). All wood sections without WGA staining showed little evidence of fluorescing hyphae, but fluorescent hyphae were observed on the middle radial faces of blocks. The percentages of wood cell coverage were higher on the middle radial faces than in WGA stained sections cut from the center (Table 3.1). This suggests that the traditional sectioning method may damage actively growing hyphae and may not be suitable for studying young hyphae that have less rigid cell walls. Eliminating the need for sectioning and fluorescent staining makes the GFP technique a very useful method for visualizing young hyphae in wood.

The intensity of fluorescence in fungal hyphae may decrease with age due to declines in cellular metabolism (van West *et al.*, 1999; Czymmek *et al.*, 2002). Hyphae with less intense fluorescence were observed occasionally on block surfaces after 4 weeks, indicating an aging of the superficial fungal hyphae. Interestingly, brightly fluorescing hyphae were commonly found at the bases of black synnemata even at 10 weeks, which may suggest that the fungus concentrated its energy to support the *Graphium* state, which produces the conidia that enable regeneration (Figure 3.6). The spore heads were also brightly fluorescent. Similar phenomena were also reported by Padgett *et al.* (1996), who found the bright fluorescence in the leaf cells infected by a *gfp* transformed virus reflected the high activity. The brightly fluorescent synnemata base hyphae were not reported by Lee *et al.* (2002), although they did observe fluorescent spore heads. This difference may be due to the use of different *O. piceae* transformants in two studies, which may have different levels of GFP expression that result in different fluorescence levels in fungal hyphae. A severe photobleaching phenomenon was encountered with their *O. piceae* transformants (personal communication), but this was not a concern for our transformants which have high photostability. No early growth of the *gfp* transformed *O. piceae* was reported in their study. We suspect that the GFP expression might be lower in their transformant, but a direct comparison study will be needed to draw any conclusions.

Table 3.1: Surface and internal assessments of Douglas-fir sapwood blocks 4 and 10 weeks after inoculation with non-transformed and *gfp* transformed *O. piceae*

Fungal Strains	4 weeks incubation				10 weeks incubation			
	Degree of Discoloration (%)		Internal Wood Cell Colonization (%)		Degree of Discoloration (%)	Internal Wood Cell Colonization (%)		
	Block		Thin Section		Block	Block	Thin Section	
	Surface ¹	Middle radial face	GFP visualized	WGA visualized	Middle radial face	Middle radial face	GFP visualized	WGA visualized
<i>O. piceae</i> (non-transformed)	25 (9) ²	68 (10)	N/A	37 (11)	77 (3)	N/A	N/A	53 (6)
OPGFI	23 (6)	75 (5)	< 1	33 (14)	70 (17)	63 (10)	0	57 (12)
OPGFII	33 (6)	73 (15)	< 1	37 (10)	78 (4)	77 (11)	0	58 (25)
OPGFIII	33 (10)	66 (20)	< 1	31 (14)	73 (6)	70 (17)	< 1	67 (6)

¹ No difference was observed for surface stain between 4 and 10 week assessments.

² Values represent mean of three replicates with the standard deviation in parenthesis.

Direct microscopic observation of the internal surfaces of wood blocks was not carried out in the first assessment at 4 weeks because it was assumed that the wood sections cut immediately from the wood blocks could provide similar information. We later noted significant differences between observations on thin radial wood sections and the middle radial faces (Table 3.1). Fluorescent hyphae were less abundant in the thin wood sections than on the exposed radial block faces. Most of the brightly fluorescing hyphae present in wood sections were fragments, suggesting that these younger hyphae may have been damaged during sectioning because the cell walls were less rigid.

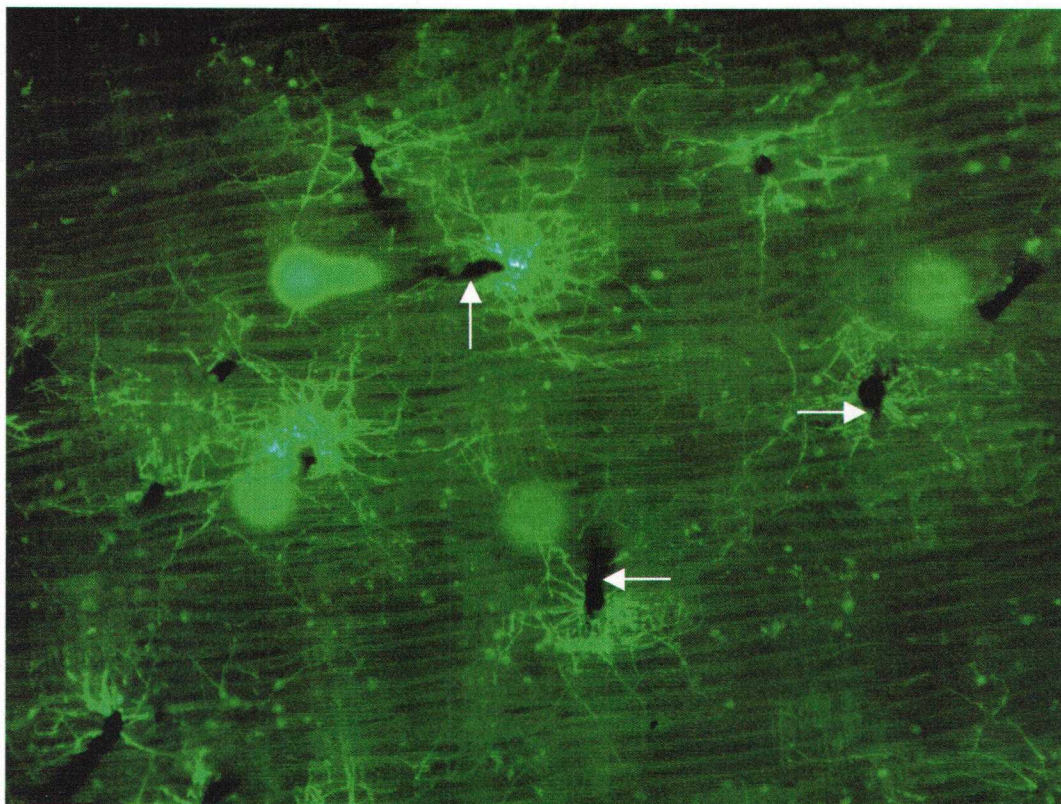


Figure 3.6: Brightly fluorescent *O. piceae* hyphae surrounding synnemata (arrows) 7 days after inoculation (Bar: 100 μ m).

***T. harzianum*:** Observations from destructive assessments carried out after 4 and 10 weeks were easier with *T. harzianum* since they only involved the assessment of spore coverage on block surfaces and internal growth. No statistical differences in growth were found between non-transformed and transformed *T. harzianum* for both superficial and internal growth (Table 3.2). Levels of fungal hyphae in WGA stained wood sections at 10 weeks decreased from those found after 4 weeks, suggesting possible cell wall disintegration in older hyphae. Higher percentages of wood cell colonization were observed on the middle radial faces

than in the WGA stained sections. These trends were similar to those observed with *O. piceae*, and further support the suggestion that traditional sectioning may not be appropriate for studying colonization of wood by younger hyphae.

Table 3.2: Surface and internal assessments of Douglas-fir sapwood blocks 4 and 10 weeks after inoculation with non-transformed and *gfp* transformed *T. harzianum*

Fungal Strains	4 weeks incubation			10 weeks incubation		
	Spore Coverage (%)	Internal Wood Cell Colonization (%)		Internal Wood Cell Colonization (%)		
	Block Surface	Thin Section		Block	Thin Section	
		<i>GFP</i> visualized	<i>WGA</i> visualized	<i>Middle radial face</i>	<i>GFP</i> visualized	<i>WGA</i> visualized
<i>T. harzianum</i> (non-transformed)	23 (3) ¹	N/A	70 (10)	N/A	N/A	60 (10)
THGFI	18 (12)	< 2	77 (3)	75 (5)	<2	65 (5)

¹ Values represent mean of three replicates with the standard deviation in parenthesis.

CONCLUSIONS

The pCT74 vector appeared to effectively transform both *O. picea*, and *T. harzianum*. Growth of transformants was similar to the non-transformed strains for both species. Transformation did not adversely affect the ability of either fungus to colonize wood, cause discoloration or sporulate. Surface growth of the transformed fungi on wood was easily monitored in situ. The resulting

transformants should prove useful for assessing the growth of young hyphae in wood, although the use of thin sections to study internal growth patterns may be problematic.

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Chapter 4: Effects of Prior Establishment of *Trichoderma harzianum* on *Ophiostoma piceae* Growth in Freshly Sawn Douglas-fir Sapwood

Ying Xiao, Jeffrey J. Morrell and Lynda M. Ciuffetti

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Effects of Prior Establishment of *Trichoderma harzianum* on *Ophiostoma piceae* Growth in Freshly Sawn Douglas-fir Sapwood

Ying Xiao¹, Jeffrey J. Morrell¹ and Lynda M. Ciuffetti²

¹ Dept. of Wood Science and Engineering, ² Dept. of Botany and Plant Pathology

Oregon State University, Corvallis, OR 97330, U.S.A.

ABSTRACT

Trichoderma harzianum has been shown to be an effective biocontrol agent against a number of wood inhabiting fungi under laboratory conditions, but this fungus has performed poorly in field trials. Understanding the interactions between biocontrol agents and their intended targets in wood may provide important clues for developing improved approaches to biocontrol, potentially reducing our reliance on pesticides. One particularly difficult problem with studying biocontrol in wood is the inability to accurately resolve individual organisms as they interact. The opaque nature of wood makes it difficult to observe interactions more than a few cells from the surface. Wood can be cut into thin sections, but the structural similarity of many fungal hyphae makes it difficult to determine which fungus is present. Transformation using genes for the synthesis of green fluorescent protein (GFP) has proven useful for visualizing specific microorganisms in their hosts and could be useful for biocontrol studies. In this study, interactions between *gfp* transformed *Ophiostoma piceae* and non-transformed *T. harzianum* were studied on freshly sawn Douglas-fir sapwood blocks. Growth of *O. piceae* was

significantly retarded when applied to wood blocks where *T. harzianum* was well-established. Interestingly the *Graphium* state of *O. piceae* was still observed on surfaces of the blocks, but the fungus was unable to penetrate deeply into the wood. The results suggest that failure of the biocontrol may not represent an inability to protect the wood beneath the surface and implies that a more detailed study of the causes of previous failures would be useful.

Key Words: biocontrol, interactions, green fluorescent protein (GFP), Douglas-fir sapwood, *Trichoderma harzianum*, *Ophiostoma piceae*.

INTRODUCTION

Wood is prone to the attack from microorganisms at moisture contents above the fiber saturation point. Sapstain fungi discolor sapwood, causing millions of dollars in losses annually (Scheffer, 1973; Anderson *et al.* 2002). The primary approach to controlling fungal discoloration has traditionally been chemical treatment shortly after sawing. However, increasing environmental concerns have led to a search for alternative methods. One alternative, biocontrol, has received considerable attention (Morrell and Sexton, 1990; Behrendt *et al.*, 1995; McAfee and Gignac, 1996; Ejечи, 1997; Kay, 1997; Sutherland and Brasier, 1997; Schoeman *et al.*, 1999; Score *et al.*, 1998; White-McDougall *et al.*, 1998; Brown

and Bruce, 1999; Brown *et al.*, 1999; Smouse *et al.*, 1999; Yang and Rossignol, 1999; McAfee *et al.*, 2001). *Trichoderma harzianum* has been shown to be an effective biocontrol agent against a number of wood inhabiting fungi under laboratory conditions, but this fungus has performed poorly in field trials (Freitag *et al.*, 1991). There are a variety of questions concerning how biocontrol agents function in wood including how these fungi grow through wood, how they interact with the target fungi, and how long they protect the wood. Understanding the interactions between biocontrol agents and their intended targets in wood may provide important clues for developing improved approaches to biocontrol, potentially reducing our reliance on pesticides. One particularly difficult problem with studying biocontrol in wood is the inability to accurately resolve individual organisms as they interact. The opaque nature of wood makes it difficult to observe interactions more than a few cells from the surface. Wood can be cut into thin sections, but the structural similarity of many fungal hyphae makes it difficult to determine which fungus is present. Fungal hyphae can be stained with fluorescent-coupled probes such as fluorescent-coupled wheat germ agglutinin, but even these compounds are relatively non-specific (Morrell *et al.*, 1985; Xiao *et al.*, 2000).

Green fluorescent protein (GFP) has been successfully used as a biomarker in many microorganisms since the cDNA for the *gfp* gene was first cloned in 1992 from the jelly fish *Aequorea victoria* (Lorang *et al.*, 2001). Microorganisms transformed with *gfp* are distinctly fluorescent, and can be easily located in their

hosts using fluorescence microscopy. Transformation of fungi with *gfp* genes has proven to be useful for understanding microbe-plant interactions and the fundamental aspects of biocontrol (Maor *et al.*, 1998; Bermudez *et al.*, 1999; Bottin *et al.*; 1999; Inglis *et al.*, 1999; Tombolini *et al.*, 1999; Stuurman *et al.*; 2000; Lorang *et al.*, 2001; Sexton and Howlett, 2001). In this report, we describe studies of the interactions between transformed *Ophiostoma piceae* (Münch) Syd. & P. Syd., and non-transformed *Trichoderma harzianum* Rifai in Douglas-fir sapwood.

MATERIAL AND METHODS

O. piceae isolate AU135-1 isolated from white spruce was kindly provided by Dr. Adnan Uzunovic at Forintek, Canada Corp (Vancouver, B. C. Canada). *T. harzianum* isolate 15B isolated from Douglas-fir roots was supplied by the U.S.D.A. Forestry Science Laboratory in Corvallis, Oregon. These fungi were transformed with a *gfp* gene as previously described (Xiao *et al.*, 2003). Isolates OPGF-I and THGF-I were two of the *gfp* transformants produced from *O. piceae* and *T. harzianum*, respectively. Freshly sawn Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) sapwood lumber was cut into wood blocks (10 × 20 × 40 mm) and sterilized by exposure to 2.5 Mrads of ionizing radiation from a cobalt 60 source.

Fungal Inoculation

Forty-two blocks were dipped for 15-20 seconds in a *T. harzianum* spore suspension containing 1×10^7 spores per mL. Excessive solution was absorbed from a corner of each sample using a sterile cotton swab. The inoculated blocks were then put onto glass rods in glass petri dishes containing moistened filter papers on the bottom. Six of these blocks were immediately treated by adding two hundred μ l of *O. piceae* OPGF-I spore suspension (1×10^7 spore per mL) to the surface of each wood. Additional sets of six blocks were inoculated with *O. piceae* in the same manner 2, 4, 6 or 8 days after *T. harzianum* treatment (Table 4.1). Moisture in the petri dishes was replenished periodically. All blocks were incubated at room temperature (20-23 °C) in the dark. Additional sets of six blocks were treated with only OPGF-I or THGF-I to serve as controls. The wood blocks inoculated with OPGF-I only were referred to as *O. piceae* control blocks, while blocks inoculated with THGF-I only were referred to as *T. harzianum* control blocks.

Assessment

The surfaces on each block were observed daily using a Nikon Elipse E400 microscope equipped with a green fluorescence filter set (excitation: 465-495nm, emission: 515-555nm). Destructive assessments were carried out after 1 or 6

weeks of incubation (Xiao *et al.*, 2003). A wood block was cut in half at the middle of the radial face to expose the middle tangential face. One half of each sample was then cut in the middle of the tangential face to expose the middle radial face, then the surfaces of the smoothed middle radial faces were directly examined using fluorescence microscopy. The patterns of fungal growth, the degree of colonization and the nature of any fungal interactions were noted.

Table 4.1: Time intervals between application of *T. harzianum* and *O. piceae* to Douglas-fir sapwood samples

Treatment	<i>T. harzianum</i> 15B	OPGF-I (<i>gfp</i> transformant)	Delay Time ¹ (days)
<i>O. piceae</i> control	–	+	N/A
0 delay	+	+	0
2-day delay	+	+	2
4-day delay	+	+	4
6-day delay	+	+	6
8-day delay	+	+	8
<i>T. harzianum</i> control	THGF-I (<i>gfp</i> transformant)	–	N/A

¹ Time between *T. harzianum* and *O. piceae* inoculation

RESULTS AND DISCUSSION

Surface Observation

One *gfp* transformed *O. piceae* strain, OPGF-I, was used in all treatments, and is referred to as *O. piceae*. For *T. harzianum*, only the control blocks were

treated with the *gfp* transformed strain (THGF-I), while dual treatments received the non-transformed *T. harzianum* strain.

Fluorescence microscopy showed that *gfp* transformed *O. piceae* spore germination on the control blocks was comparable to that found on artificial media (Xiao *et al.*, 2003). Spore germination was abundant on the wood surface after 22 hrs, and fungal growth covered the wood surface after 2 days. Pigmented synnemata with spore heads started to emerge on the fourth day. Well-developed, black synnemata with spore heads covered all wood blocks by the fifth day. *O. piceae* spore germination and formation of black synnemata in the presence of *T. harzianum* when both fungi were simultaneously inoculated were similar to that discovered on *O. piceae* control blocks.

O. piceae spore germination was notably lower on wood blocks treated with a 2-day delay than on *O. piceae* controls after 22 hrs. Black synnemata also formed more slowly than on *O. piceae* control blocks. Most synnemata lacked spore heads by the fifth day. The number of black synnemata was lower and the spore heads were smaller than those on control blocks after 11 days (Figure 4.1).

Spore germination on wood blocks treated with a 4-day delay was very sparse after 22 hrs, and was patchy on the third day. Synnemata were largely non-pigmented on the fifth day. Black synnemata were noticeable after 11 days, but had smaller spore heads than those produced on blocks treated with a 2-day delay (Figure 4.1). Brightly-fluorescent hyphae surrounding black synnemata were

observed after 6 weeks, but appeared to be less abundant than those found on control blocks.

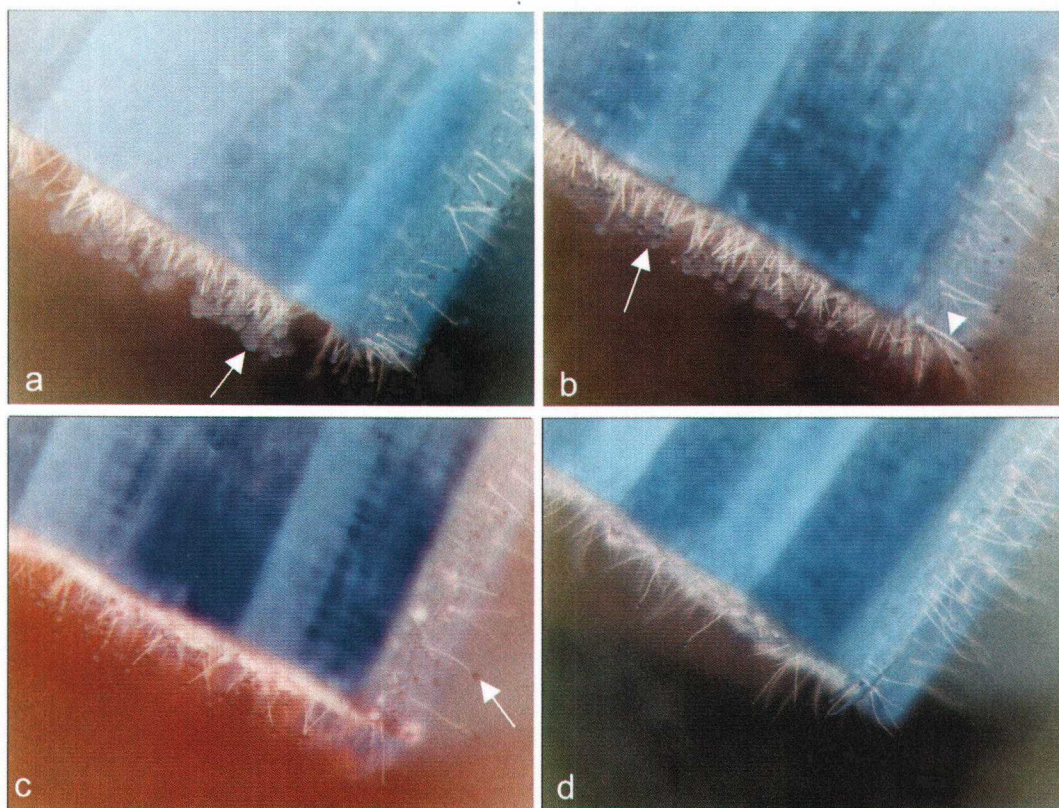


Figure 4.1: Stereo light micrographs showing appearance of *O. piceae* synnemata on wood blocks 11 days after inoculation with: a) *O. piceae* alone or *T. harzianum* followed by *O. piceae* with a b) 0 delay; c) 2-day delay; or d) 4-day delay. Images were inverted so that black synnemata stalks became white (arrow head), and spore heads became darker (arrows).

O. piceae growth was very similar on blocks treated with 6- and 8-day delay treatments. Spore germination was absent until the third day, and only a few black synnemata formed after 7 days. Black synnemata were more abundant after 6 weeks, but the distribution was not uniform on the wood surfaces. Bright hyphae

surrounding black synnemata were observed after 6 weeks, but were less frequent than those found on blocks treated with a 4-day delay.

Sporulation of *T. harzianum* was similar on all *T. harzianum* treated wood blocks except those receiving both fungi simultaneously where little *T. harzianum* sporulation was observed. Scattered light green spore masses were observed on blocks 7 days after *T. harzianum* inoculation. The areas covered by spore masses appeared to stabilize around the tenth day, then spores developed a dark green color.

Assessment

Sporulation and internal growth of the non-transformed and transformed *T. harzianum* were comparable (Xiao *et al.*, 2003). Therefore, control blocks were treated with the *gfp* transformed strain (THGF-I) since it was easier to visualize the growth of the transformant. Destructive assessment 1 week after inoculation showed that *T. harzianum* grew better inside the wood blocks than *O. piceae* (Table 4.2), although spore germination was slower than that of *O. piceae* (Xiao *et al.*, 2003). Both *T. harzianum* and *O. piceae* completely colonized their respective control blocks after six weeks. However, more fluorescent hyphae were observed in wood cells at the two ends than the middle of the blocks colonized by *O. piceae*.

O. piceae penetration in blocks treated simultaneously with the two test fungi appeared to be deeper than that in the control blocks after 1 week. Penetration of *O. piceae* hyphae in simultaneously treated blocks was thorough

and uniformly distributed in blocks after 6 weeks, in contrast to the relatively sparse distribution in the middle parts of *O. piceae* control blocks. *T. harzianum* spore germination was slower than *O. piceae* on Douglas-fir sapwood, giving *O. piceae* a competitive advantage over *T. harzianum*. The presence of *T. harzianum* may have also stimulated *O. piceae* growth. *T. harzianum* did not show normal sporulation on blocks treated simultaneously with the two fungi (Figure 4.2), suggesting that *T. harzianum* growth may have been inhibited by *O. piceae*. The differences in *O. piceae* growth alone and in the presence of the antagonist highlights the importance of early establishment for control of an organism.

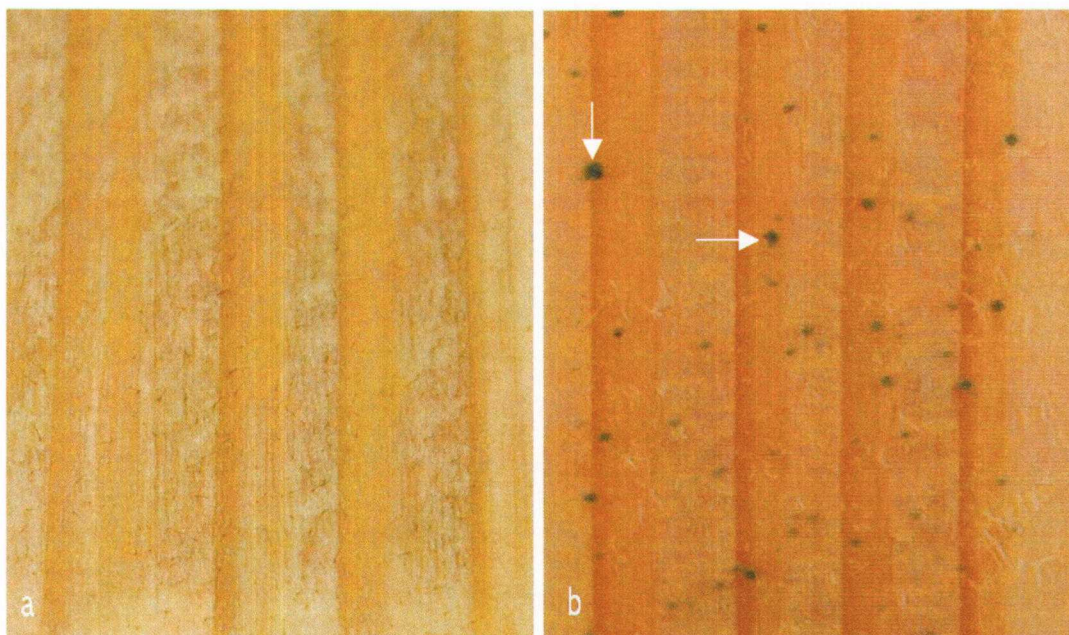


Figure 4.2: Douglas-fir sapwood surfaces a) simultaneously treated with *T. harzianum* and *O. piceae* and incubated for 12 days or b) treated with *T. harzianum*, then *O. piceae* with a 4-day delay and incubated for 9 days. Arrows show masses of *T. harzianum* spores.

Two out of three blocks treated with a 2-day delay showed similar degrees of *O. piceae* penetration (Table 4.2), but wood cell colonization was lower (data not shown). No fluorescing hyphae were observed in the third block after 1 week.

High variations in growth patterns were observed between replicates after 6 weeks. Fluorescing hyphae were detected in about 5 percent of wood cells in the middle part of one block, but were widely scattered in ray cells and tracheids of the other two blocks. Germination of *T. harzianum* began after 2 days of incubation at about the same time that *O. piceae* was inoculated onto the blocks. Competition produced a high degree of variation in fungal growth inside this set of blocks. *T. harzianum* appeared to be a slower germinator, but a faster grower once it germinated. *O. piceae* growth was less abundant in the 2 day delay blocks compared to the controls, suggesting that *T. harzianum* was effective soon after its germination.

Although black synnemata and bright green hyphae were observed on the surfaces of blocks treated with *O. piceae* 4-, 6-, or 8-days after application of *T. harzianum*, almost no fluorescent hyphae were observed inside the blocks after 1 week (Figure 4.3). After 6 weeks, fluorescing hyphae were occasionally observed in all blocks treated with 4 to 8 day delays between *T. harzianum* and *O. piceae* application. The level of colonization was rather sparse (one to ten hyphae per block), and suggested that *T. harzianum* was inhibiting *O. piceae* colonization.

Table 4.2: Depth of longitudinal hyphal penetration and percentage of wood cell colonization 1 or 6 weeks after *O. piceae* inoculation (see Table 4.1 for treatments)

Treatment	1 week		6 weeks	
	Longitudinal Penetration Depth (cm)	Internal Wood Cell Colonization ¹ (%)	Longitudinal Penetration Depth (cm)	Internal Wood Cell Colonization ¹ (%)
OPGF-I	0.9 (0.2) ²	19 (3)	2	27 (3)
0 delay	1.3 (0.2)	25 (4)	2	43 (6)
2-day delay	0.9 (0.7)	5 (4)	N/A	<1
4-day delay	N/A	<1	N/A	<1
6-day delay	N/A	<1	N/A	<1
8-day delay	N/A	<1	N/A	<1
THGF-I	1.2 (0.4)	26 (8)	2	68 (13)

¹ Values represent % of cells colonized by fluorescent *O. piceae* at a given time.

² Values represent means of 3 replicates with the standard variations in the parenthesis.

The degree of *O. piceae* growth (both superficial and internal) tended to be inversely related to the delay time. The frequency of fluorescent hyphae at the bases of the black synnemata also decreased when the delay time increased, implying that growth stresses for *O. piceae* increased when *T. harzianum* was better established in the wood. These results illustrate how the degree of establishment of *T. harzianum* in the wood determines its effectiveness against *O. piceae*.

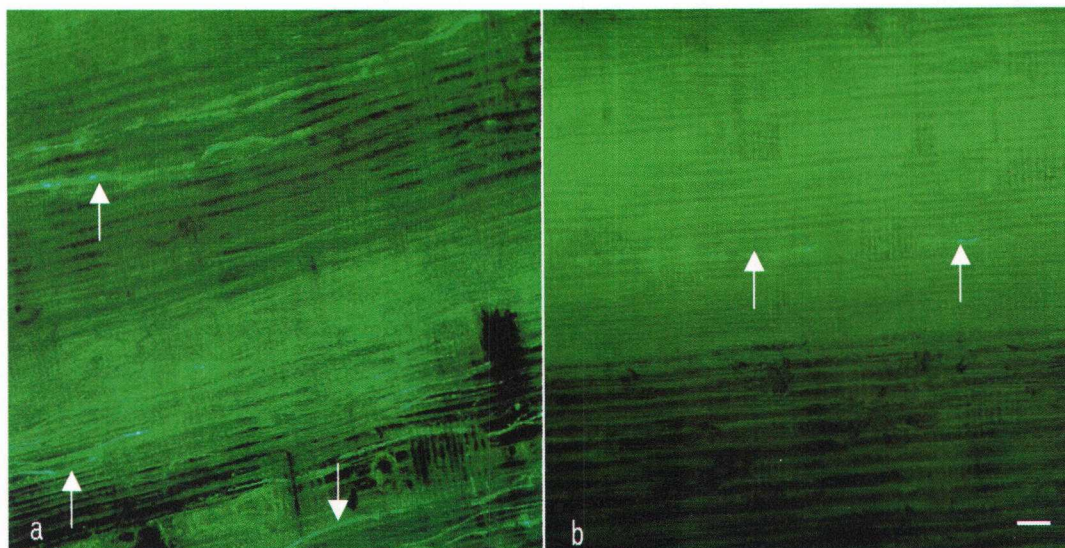


Figure 4.3: Fluorescence micrographs showing a) fluorescent hyphae in an *O. piceae* control block at the longitudinal end region (arrows) or b) fluorescent hyphae in 4-day delay treated wood block 1 week after inoculation (Bar: 100 μ m).

It was interesting to note that *O. piceae* was able to develop the *Graphium* stage on wood surfaces inoculated with *T. harzianum*, but failed to show comparable growth inside the wood. To verify whether fluorescent hyphae were present immediately beneath the surface where fluorescent hyphae were present at the bases of synnemata, thick hand sections (100-300 μ m) were taken from the wood surface with the synnemata carefully preserved. Both the surfaces and their reverse sides were observed for fluorescing hyphae. Fluorescing hyphae were observed on the reverse surfaces of blocks treated with *O. piceae* alone as well as 0 delay and 2-day delay treatments, while no fluorescing hyphae were observed on the reverse sides of sections from the 4-, 6-, and 8-day delay treatments. The

occasional presence of fluorescing hyphae in the middle of some blocks treated with *O. piceae* after 4-, 6-, and 8-day delays indicated that *O. piceae* was still able to survive in some niches. The results indicate that established *T. harzianum* clearly inhibits the growth of *O. piceae* inside the wood, but is less effective on the surfaces. Stain control is largely a surface phenomenon. Therefore, *T. harzianum* may not be an ideal biocontrol agent against *O. piceae* in freshly sawn Douglas-fir sapwood since it cannot completely limit growth on the wood surface.

CONCLUSIONS

Although *T. harzianum* spores germinated more slowly than those of *O. piceae* on fresh Douglas-fir sapwood, the former fungus colonized the wood more rapidly once it germinated. Simultaneous inoculation or slight delays between *T. harzianum* and *O. piceae* introduction allowed the stain fungus to grow normally. *O. piceae* growth was limited when *T. harzianum* was well established, while *T. harzianum* was unable to sporulate on wood when *O. piceae* was inoculated at the same time. These results suggest that both fungi were antagonistic to one another. The partial protection and importance of internal colonization by the biocontrol fungus partly explain the failures reported in field tests and suggest the need for further trials to better understand the role of initial colonization on biocontrol performance.

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**Chapter 5: Effect of Spore Ratio on the Biocontrol Efficacy of
Trichoderma harzianum against *Ophiostoma piceae* in Douglas-fir
Sapwood**

Ying Xiao, Jeffrey J. Morrell and Lynda M. Ciuffetti

Effect of Spore Ratio on the Biocontrol Efficacy of *Trichoderma harzianum* against *Ophiostoma piceae* in Douglas-fir Sapwood

Ying Xiao¹, Jeffrey J. Morrell¹ and Lynda M. Ciuffetti²

¹ Dept. of Wood and Science Engineering, ² Dept. of Botany and Plant Pathology

Oregon State University, Corvallis, OR 97330, U.S.A.

ABSTRACT

Understanding the interactions between biocontrol agents and their intended targets in wood may provide important clues for developing improved approaches to biocontrol, potentially reducing our reliance on pesticides. However, the structural similarity of many fungal hyphae makes it difficult to resolve individual hyphae and assess fungal interactions in wood. A green fluorescent protein (GFP) technique allows *in situ* visualization of a microorganism and was used to study the interactions of *Trichoderma harzianum*, a potential biocontrol agent, and *Ophiostoma piceae*, a common sapstain fungus, in wood. Freshly sawn Douglas-fir sapwood blocks were inoculated with a spore suspension containing spores of a *gfp* transformed *O. piceae* and a non-transformed *T. harzianum* at different ratios. The ability of *T. harzianum* to inhibit *O. piceae* was enhanced by increasing the levels of *T. harzianum* spores. The possible modes of action and practical implications towards improving biocontrol efficacy are discussed.

Key Words: conidiospores, *Trichoderma harzianum*, *Ophiostoma piceae*, sapstain, biocontrol, green fluorescent protein.

INTRODUCTION

Sapstain fungi discolor sapwood, causing millions of dollars in losses annually (Scheffer, 1973; Anderson *et al.*, 2002). *Ophiostoma piceae* (Münch) Syd. & P. Syd. is an important sapstain fungus of conifers in the Pacific Northwest. Damage by this fungus is typically controlled by application of fungicides to the wood, but there are increasing public concerns about the use of pesticides. An environmentally benign alternative to the conventional chemical approach for controlling sapstain fungi is to use biocontrol to limit sapstain fungi (Morrell and Sexton, 1990; Behrendt *et al.*, 1995; McAfee and Gignac, 2001; Ejechi, 1997; Kay, 1997; Sutherland and Brasier, 1997; Schoeman *et al.*, 1999; Score *et al.*, 1998; White-McDougall *et al.*, 1998; Brown and Bruce, 1999; Brown *et al.*, 1999; Smouse *et al.*, 1999; Yang and Rossignol, 1999; McAfee *et al.*, 2001). Strains of *Trichoderma* spp. have long been of interest as biocontrol organisms against wood decay fungi (Shields and Atwell, 1963). One species, *Trichoderma harzianum* Rifai, has been reported as a potential biocontrol agent of *O. piceae*; however, it has not been commercialized due to its highly variable field performance (Freitag *et al.*, 1991). The reasons for these failures are difficult to

determine due to the inability to resolve individual fungal hyphae in a fungal consortium on wood.

Green fluorescent protein (GFP) techniques insert a *gfp* gene into a microorganism that, when expressed, imparts an autofluorescence to this microorganism. The GFP technique is useful for studying specific microorganisms *in situ*, and has been widely applied in various biological fields (Lorang *et al.*, 2001). Transformation of *T. harzianum* and *O. piceae* with *gfp* gene has proven to be useful for studying the interactions of these two fungi in wood (Xiao *et al.*, 2003a; Xiao *et al.*, 2003b).

T. harzianum tends to germinate more slowly than *O. piceae* on fresh Douglas-fir sapwood, which may partially account for the field failures with this fungus (Xiao *et al.*, 2003b). Prior establishment of *T. harzianum* appeared to increase the effectiveness of this fungus against *O. piceae*. *O. piceae* and *T. harzianum* are antagonistic to each other, and the species that germinates first will tend to dominate the substrate. One approach to enhancing successful *T. harzianum* colonization may be to apply an overwhelming number of spores. In this study, the effect of the ratio of spore of the biocontrol agent to the target fungus on the biocontrol efficacy was investigated using a *gfp* transformed *O. piceae* and a non-transformed *T. harzianum* in Douglas-fir sapwood.

MATERIAL AND METHODS

O. piceae isolate AU135-1 isolated from white spruce was kindly provided by Dr. Adnan Uzunovic at Forintek, Canada Corp (Vancouver, B. C. Canada). *T. harzianum* isolate 15B isolated from Douglas-fir roots was supplied by the U.S.D.A. Forestry Science Laboratory in Corvallis, Oregon. These fungi were transformed with a *gfp* gene as previously described (Xiao *et al*, 2003). Isolates OPGF-I and THGF-I were two of the *gfp* transformants produced from *O. piceae* and *T. harzianum*, respectively. Freshly sawn Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) sapwood lumber was cut into wood blocks (10 × 20 × 40 mm in the grain direction) and sterilized by exposure to 2.5 Mrads of ionizing radiation from a cobalt 60 source.

Fungal Inoculation

Thirty-nine blocks were dipped for 15-20 seconds in a spore suspension containing non-transformed *T. harzianum* and *gfp* transformed *O. piceae* OPGF-I spores at different ratios (Table 5.1). Excessive solution was absorbed from a corner of each sample using a sterile cotton swab. The inoculated blocks were then put onto glass rods in glass petri dishes containing moistened filter papers on the bottom. Moisture in the petri dishes was replenished periodically. All blocks were incubated at room temperature (20-23 °C) in the dark. A reference set of wood

blocks were treated similarly with *gfp* transformed *T. harzianum* THGF-I and non-transformed *O. piceae*. Control wood blocks were inoculated with OPGF-I at a concentration of 10^7 spores/mL. There were nine replicates for each treatment.

Table 5.1: Number of spores per mL of spore suspension applied to Douglas-fir sapwood blocks

Treatment	<i>T. harzianum</i> 15B (non-transformant)	OPGF-I (<i>gfp</i> transformed)	THGF-I (<i>gfp</i> transformant)	<i>O. piceae</i> AU 135-1 (non-transformed)
Control	–	10^7	–	–
TH:OP (1:1)	10^7	10^7	–	–
TH:OP (10:1)	10^7	10^6	–	–
TH:OP (100:1)	10^7	10^5	–	–
TH:OP (1000:1)	10^8	10^5	–	–
Reference	–	–	10^7	10^7

Assessment

The upper surfaces on each block were observed periodically using a Nikon Elipse E400 microscope equipped with a green fluorescence filter set (excitation: 465-495nm, emission: 515-555nm). Spore germination, hyphal growth and synnemata development on each specimen were observed in five 5mm diameter fields using a 4× objective lens (Figure 5.1).

Three replicates were destructively sampled for internal assessment after 1, 2, or 6 weeks of incubation (Xiao *et al*, 2003a). Each wood block was cut in half lengthwise along the middle of the radial face to expose the middle tangential face.

One half of each sample was then cut at middle of the tangential face to expose the middle radial face, then the surfaces of the smoothed middle radial faces were directly examined using fluorescence microscopy. The patterns of fungal growth, the degree of colonization and the nature of any fungal interactions were noted.

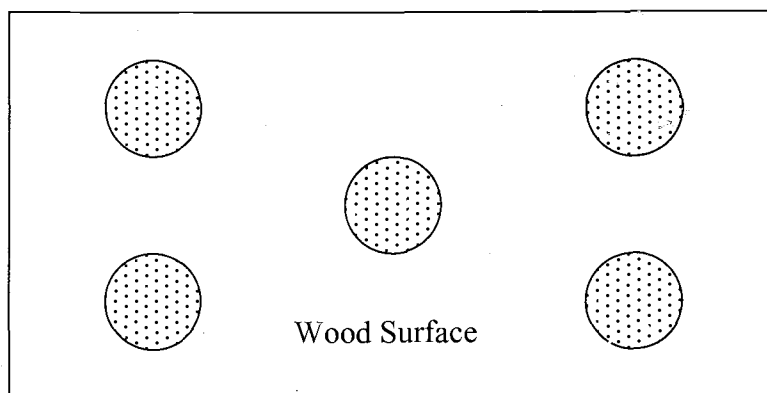


Figure 5.1: Locations of the five 5mm diameter fields observed with a 4 \times lens on Douglas-fir sapwood inoculated with combinations of *T. harzianum* and *O. piceae*.

Data Analysis

O. piceae spore germination, hyphal development, and synnemata development were compared using Tukey's HSD multiple comparison method or a two-sample t-test on SAS (SAS ver 8.2, SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

In a preliminary test, *O. piceae* was inoculated onto wood at different densities. At the early growth stages, *O. piceae* hyphal development was faster when spore density was lower on wood surface, which may be due to better access to nutrient source for individual hypha or less self-inhibition (lab observation, not published). With time *O. piceae* hyphal development and synnemata development appeared to be similar in those treatments. Therefore, spore density of *O. piceae* did not have a big effect its growth on wood. Similar observations were also made with *Gloeophyllum trabeum*, in a previous study (Schmidt and French, 1979).

In this study, *O. piceae* spore germination and hyphal development were inversely related to the TH:OP spore ratio (Figure 5.2). Spore germination of *O. piceae* on the control, TH:OP (1:1), and TH:OP (10:1) treated blocks was too heavy to count 24 hrs after inoculation. The number of germinating spores on TH:OP (100:1) treated blocks was significantly higher than that on TH:OP (1000:1) treated blocks 24 hrs after inoculation (p -value = 0.0002 at a 0.05 significant level), and increased even more 48 hrs after inoculation (p -value < 0.0001 at a 0.05 significant level) (Figure 5.3). In contrast, there was little difference in spore germination on TH:OP (1000:1) treated blocks 24 or 48 hrs after inoculation. The TH:OP (100:1) and TH:OP (1000:1) treatments used the same volume of *O. piceae*, but the number of *T. harzianum* spores differed (Table

5.1). Increasing the proportion of *T. harzianum* appeared to inhibit *O. piceae* spore germination.

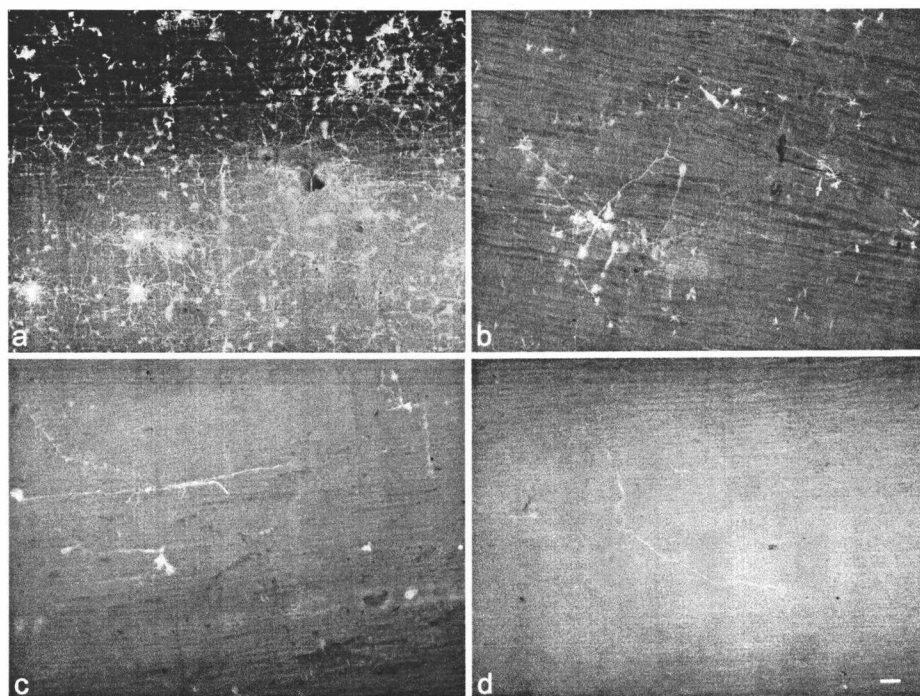


Figure 5.2: *O. piceae* spore germination and hyphal growth 48 hrs after inoculation with selected combinations of *O. piceae* (OP) and *T. harzianum* (TH): a) TH:OP (1:1) b) TH:OP (10:1) c) TH:OP (100:1) d) TH:OP (1000:1) (Bar: 100 μ m).

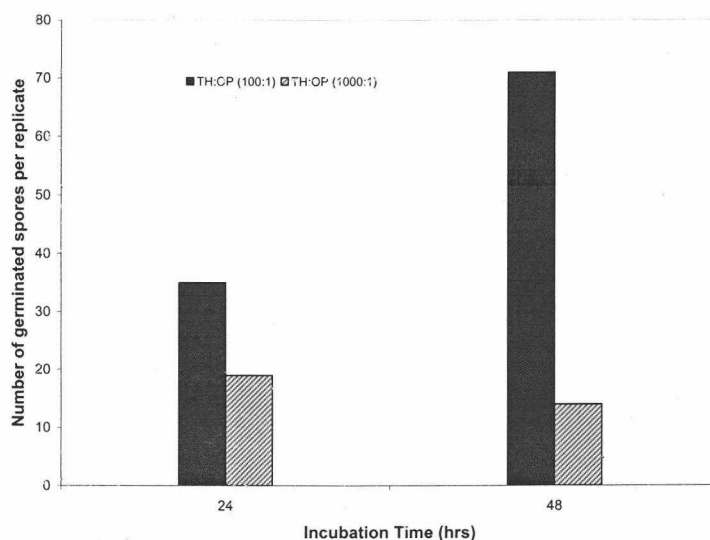


Figure 5.3: Number of *O. piceae* spores that germinated on wood surfaces 24 or 48 hours after inoculation with combinations of spores of *O. piceae* and *T. harzianum* (average number of nine replicates).

Numerous brightly-fluorescent synnemata emerged two days after inoculation on the wood surface of the control, TH:OP (1:1), and TH:OP (10:1) treatments, although they were still invisible to the naked eye. Pigmentation developed in these synnemata three days after inoculation, but spore heads were not yet evident (Table 5.2). Pigmented synnemata were not observed at this stage for the TH:OP (100:1) and TH:OP (1000:1) treatments. Darkly pigmented synnemata became visible to the naked eye by the fourth day. The number of pigmented synnemata on wood blocks after 4 days was comparable for the control, reference, and TH:OP (1:1) treatments and spore heads were present on the majority of the synnemata (Table 5.2) (Table 5.3). Spore-head development appeared to be inversely related to the initial spore ratio at this stage; however, differences between treatments became smaller as incubation time increased. The

development of synnemata and spore heads plateaued after 14 days of incubation for all treatments (Figure 5.4). After 42 days of incubation, the control (with no TH), TH:OP (1:1), TH:OP (10:1), and the reference treatment showed similar levels of synnemata production. The number of synnemata present on TH:OP (100:1) and TH:OP (1000:1) was significantly lower than the other four treatments (Table 5.3), suggesting that *O. piceae* synnemata development was limited at higher spore ratios.

Table 5.2: Development of *O. piceae* synnemata and spore heads 3 to 7 days after inoculation with combinations of *O. piceae* and *T. harzianum*

Treatment	3 days		4 days		7 days	
	Number of Pigmented Synnemata	Synnemata with spore heads (%)	Number of Pigmented Synnemata	Synnemata with spore heads (%)	Number of Pigmented Synnemata	Synnemata with spore heads (%)
Control	88(19) ¹	0	98(18)	65(11)	101(17)	100
TH:OP (1:1)	93(19)	0	104(20)	64(14)	109(19)	100
TH:OP (10:1)	19(9)	0	83(17)	43(17)	85(18)	77(14)
TH:OP (100:1)	0	0	25(10)	7(4)	35(8)	33(8)
TH:OP (1000:1)	0	0	5(3)	0	28(9)	25(10)
Reference	100(24)	0	108(20)	65(13)	112(18)	100

¹ Values represent means of nine replicates with the standard deviation in parenthesis.

Table 5.3: Tukey's HSD analysis results for *O. piceae* hyphal and synnemata development on Douglas-fir sapwood after inoculation with combinations of *O. piceae* (OP) and *T. harzianum* (TH)

Hyphal Development		Synnemata Development	
2 days	7 days	14 days	42 days
TH:OP (1:1) a^1	TH:OP (1:1) a	Reference	Reference
Control	Control	TH:OP (1:1) a	TH:OP (1:1) a
TH:OP (10:1) b	TH:OP (10:1) b	Control	Control
TH:OP (100:1) c	TH:OP (100:1) c	TH:OP (10:1) b	TH:OP (10:1)
		TH:OP (100:1) c	TH:OP (100:1) b
		TH:OP (1000:1)	TH:OP (1000:1)

¹Treatments with the same letter are not significantly different (at a 0.05 significant level). The means for treatments are in a decreasing order from above to below.

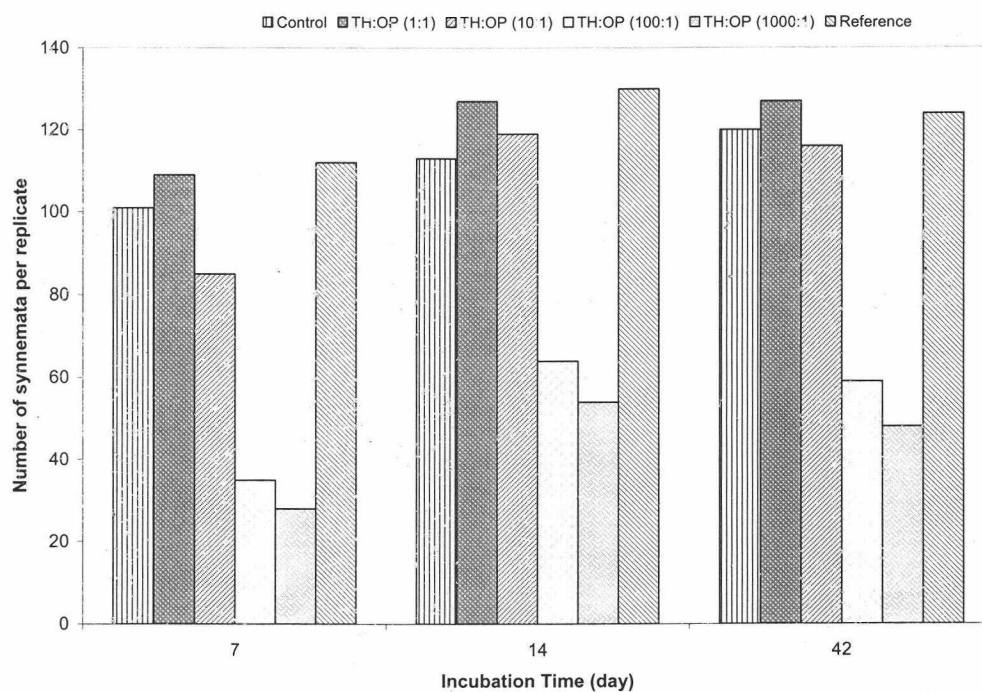


Figure 5.4: Number of pigmented *O. piceae* synnemata on the wood surface 1, 2, or 6 weeks after inoculation with combination of *O. piceae* (OP) and *T. harzianum* (TH) (average number of three replicates).

Fluorescence intensity in fungal hyphae can be indicative of the vigor of a fungus, and may decrease with age as cellular metabolism declines (van West *et al.*, 1999; Webb *et al.*, 2001 Czymmek *et al.*, 2002). The amount of fluorescent *O. piceae* hyphae on the wood surface was reduced when *T. harzianum* spore concentrations were higher than *O. piceae* (Figure 5.5). The amount of fluorescent hyphae on control blocks and TH:OP (1:1) treated blocks peaked 2 days after inoculation, then sharply declined and decreased thereafter to a stable level of between 30 – 50% of the cells after 1 week. This sharp decrease may be associated with the initiation of the *Graphium* stage, which requires most of the microbial metabolic activity (Xiao *et al.*, 2003a). Fluorescence in TH:OP (10:1) treated blocks also peaked 2 days after inoculation, then the amount of fluorescent hyphae declined to a very low level after 1 week of incubation. Synnemata and spore head development in this treatment was only slowed down compared to the control and the TH:OP (1:1) treatment (Table 5.2 and Figure 5.3). The significant decrease in the amounts of fluorescent *O. piceae* hyphae suggest that *O. piceae* was stressed in the presence of the biocontrol agent (Table 5.3). A similar but more pronounced trend was observed with the TH:OP (100:1) treatment. Note that the hyphal development for TH:OP (1000:1) treatment was not included in the statistic analysis because almost no fluorescent hyphae were observed on TH:OP (1000:1) treated blocks over the incubation period. This suggested that *T. harzianum* completely inhibited superficial hyphal development of *O. piceae* at the high spore ratio.

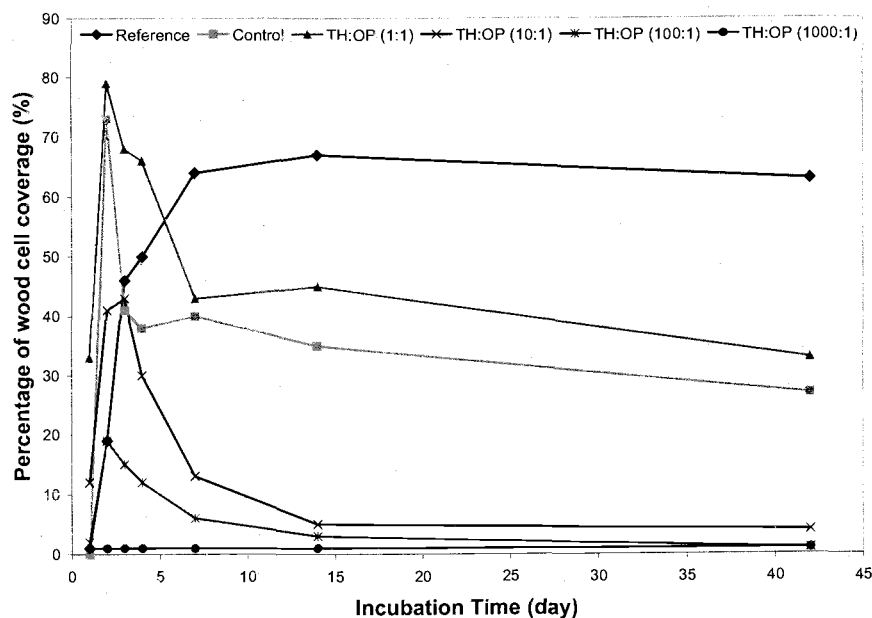


Figure 5.5: Percentage of fluorescent hyphal coverage on the surfaces of Douglas-fir blocks inoculated with combinations of *O. piceae* (OP) and *T. harzianum* (TH) (average value for three replicates).

Most brightly fluorescent hyphae surrounded the bases of the synnemata (Figure 5.6), indicating the fungus was most active in these regions. This observation is consistent with previous studies (Xiao *et al.*, 2003a & b), where the fungus appeared to concentrate metabolic activity to produce synnemata and spore heads under stress. Maximum fluorescence in some plant-microbe interaction studies using *gfp* expression has been found to be associated with infection or formation of pycnidial initials where microbial activity is likely to be the greatest (Padgett *et al.*, 1996; Rohel *et al.*, 2001; Sexton and Howlett, 2001). The association of high metabolic activities with the development of synnemata illustrated the importance of spore development. High concentrations of *T. harzianum* spores significantly reduced or diminished hyphal growth of *O. piceae*,

particularly at a very high spore ratio. The small amounts of hyphae observed for these *T. harzianum* treatments were primarily associated with synnemata development. The high growth stresses on TH:OP (1000:1) treated blocks eliminated hyphal development of *O. piceae*, but not synnemata and spore head development (Table 5.2, Figure 5.4, 5.5 & 5.6). This observation illustrates the ability of *O. piceae* to survive under adverse conditions, which may also explain the difficulty in controlling this fungus.

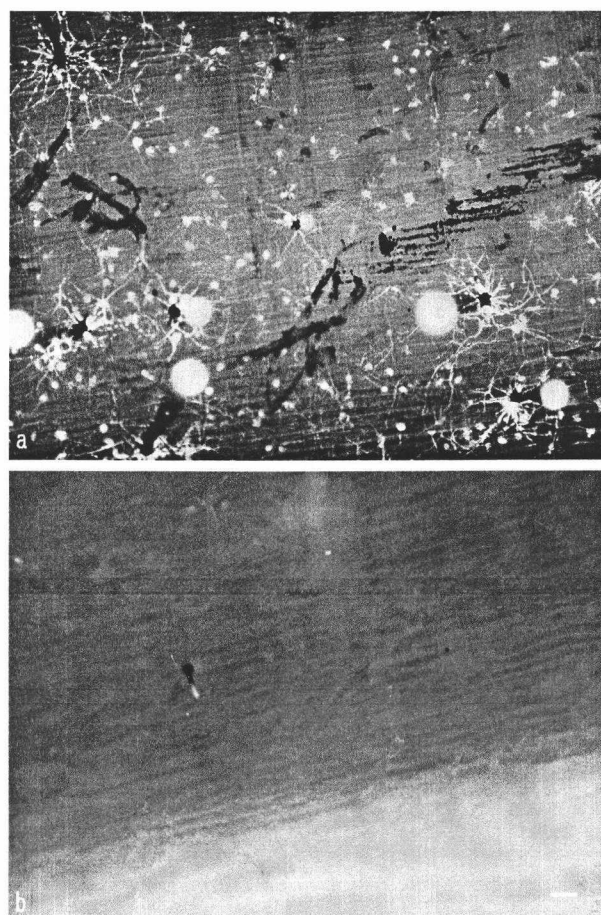


Figure 5.6: Formation of synnemata on the surface of Douglas-fir sapwood 4 days after inoculation with: a) TH:OP (1:1) or b) TH:OP (1000:1) (Bar: 100 μ m).

Internal assessment of exposed blocks showed that *O. piceae* growth was primarily in control and TH:OP (1:1) treated blocks (Table 5.4). Very few fluorescent hyphae were observed in TH:OP (10:1), TH:OP (100:1), and TH:OP (1000:1) treated blocks, suggesting that *O. piceae* was largely unable to grow inside wood colonized by *T. harzianum*. Low internal growth activity was also observed when *O. piceae* was applied to wood after *T. harzianum* grew on the wood surface (Xiao *et al.*, 2003b). The results indicate that *T. harzianum* at a 10:1 ratio had a suppression effect on *O. piceae*. Higher spore ratios enhanced this effect.

Table 5.4: Internal assessments of Douglas-fir sapwood blocks 1, 2, and 6 weeks after inoculation with combinations of *O. piceae* (OP) and *T. harzianum* (TH)

Treatment	Internal Growth (% of wood cell coverage)		
	1wk	2wks	3wks
Control	22(13) ¹	40(17)	47(15)
TH:OP (1:1)	23(8)	37(15)	53(15)
TH:OP (10:1)	<5	<2	<1
TH:OP (100:1)	<1	<5	<1
TH:OP (1000:1)	<5	<1	<1
Reference ²	21(18)	25(5)	18(10)

¹ Values represent mean of three replicates with the standard deviation in parenthesis

² The numbers for the reference treatment are for *T. harzianum* growth

Simultaneous inoculation with non-transformed *T. harzianum* and the *gfp* transformed *O. piceae* at the same concentration almost eliminated *T. harzianum*

sporulation, suggesting that both fungi were antagonistic (Xiao *et al.* 2003b). A reference trial using the *gfp* transformed *T. harzianum* and the non-transformed *O. piceae* showed that *T. harzianum* sporulation was much lower than the other applicable treatments for the control and the reference treatment (Table 5.5). These results are consistent with previous studies. The results also showed that *T. harzianum* developed abundant hyphae (Figure 5.5). The amounts of fluorescent THGF-I hyphae gradually increased over the first week, and then remained constant. In contrast to *O. piceae*, *T. harzianum* developed vegetative growth, but failed to produce spores under stress, suggesting that these fungi exhibit a different survival strategy.

Table 5.5: Surface sporulation by *T. harzianum* on Douglas-fir sapwood blocks 1, 2 and 6 weeks after inoculation with combination of *T. harzianum* and *O. piceae*

Treatment	Surface Sporulation (% of wood cell coverage)		
	1wk	2wks	6wks
Control	N/A ¹	N/A	N/A
TH:OP (1:1)	<1	<5	7(3)
TH:OP (10:1)	13(12)	26(8)	28(6)
TH:OP (100:1)	16(6)	25(8)	33(10)
TH:OP (1000:1)	23(9)	24(10)	32(10)
Reference	0	8(4)	7(6)

¹ N/A: not applicable

T. harzianum has been reported to function as a biocontrol agent by various combinations of three mechanisms: competing for nutrients, antibiosis, and mycoparasitism that vary with situation. It is important to identify its main mode of action against the target fungus for effective biocontrol. *T. harzianum* competes for non-structurally bound nutrients in wood, which will certainly affect the growth of sapstain fungi that share the same nutrients source (Hulme and Shields, 1972; Ballard et al., 1982; Blanchette et al., 1992; Fisher et al., 1994). As mentioned previously, *O. piceae* hyphal development was faster when its spore density was lower on the wood surface if no other microorganisms were present. The fact that *O. piceae* hyphal development was limited in TH:OP (10:1) and TH:OP (100:1) treated blocks in this study implies the one primary mode of action of *T. harzianum* by producing toxic metabolites. The spore concentration of *T. harzianum* was maintained at the same level in the inocula for these treatments. If the main mode of action was by nutrient depletion, *T. harzianum* would deplete nutrients similarly in these treatments and *O. piceae* growth in TH:OP (10:1) and TH:OP (100:1) treated blocks should be comparable to that in TH:OP (1:1) treated blocks. Metabolites produced by *T. harzianum* have antifungal properties and have been used for the control of wood degrading fungi (Bruce et al., 1984; Claydon et al., 1987; Bettucci et al., 1988; Avent et al., 1992; Srinivasan et al., 1992; Horvath et al., 1995; Bruce et al., 1996; Hill et al., 1997). *T. harzianum* may produce toxic metabolites during spore germination that are effective against *O. piceae*. As a

result, spore concentration is an important determinant of which organism will dominate a substrate.

While it would be difficult to control *O. piceae* spore density in the real world, it is always possible to increase the concentration of *T. harzianum* inoculum to improve efficacy. Periodic reapplication of the biocontrol agent may also help to improve effectiveness.

CONCLUSIONS

The ratio of *T. harzianum* to *O. piceae* spores had a positive effect on the biocontrol efficacy of *T. harzianum*. Inoculum containing 10:1 *T. harzianum* : *O. piceae* (TH:OP) inhibited internal growth of *O. piceae*, but did not limit the surface hyphal growth or synnemata development. *T. harzianum* inhibited both superficial and internal *O. piceae* growth when the TH:OP ratio was 100:1 or higher. These results suggest that biocontrol efficacy may be improved by increasing the spore concentration in the *T. harzianum* inoculum or by periodically reapplying *T. harzianum* spore suspensions.

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**Chapter 6: Effects of Live or Killed *Trichoderma harzianum* on
Ophiostoma piceae Growth in Freshly Sawn Douglas-fir Sapwood**

Ying Xiao, Jeffrey J. Morrell and Lynda M. Ciuffetti

Effects of Live or Killed *Trichoderma harzianum* on *Ophiostoma piceae* Growth in Freshly Sawn Douglas-fir Sapwood

Ying Xiao¹, Jeffrey J. Morrell¹ and Lynda M. Ciuffetti²

¹ Dept. of Wood and Science Engineering, ² Dept. of Botany and Plant Pathology
Oregon State University, Corvallis, OR 97330, U.S.A.

ABSTRACT

The inability to resolve hyphae of different fungi makes it difficult to assess fungal interactions in wood. A green fluorescent protein (GFP) technique allows *in situ* visualization of a microorganism and was used to study the interactions of *Trichoderma harzianum*, a potential biocontrol agent, and *Ophiostoma piceae*, a common sapstain fungus, in wood. In this study, GFP was used to examine the biocontrol efficacy of live or killed *T. harzianum* grown on wood prior to application of *O. piceae*. Fluorescent labeled lectins were not effective for staining fungal hyphae on wood block surfaces directly, and spores tended to be washed from the wood surface. In contrast, GFP allows visualization of spore germination without artificial staining, and was extremely useful for monitoring early fungal growth in wood.

Prior establishment of *T. harzianum* on freshly sawn Douglas-fir sapwood effectively inhibited *O. piceae*, but the inhibition was lost when *T. harzianum* was killed by γ -irradiation. On the other hand, when established *T. harzianum* was

killed by autoclaving, it still afforded partial protection against *O. piceae*. The results suggested that *T. harzianum* must remain alive to be most active against *O. piceae*.

Key words: *Trichoderma harzianum*, *Ophiostoma piceae*, metabolites, biocontrol, wood, sapstain, green fluorescent protein.

INTRODUCTION

Sapstain fungi discolor sapwood, causing millions of dollars in losses annually (Scheffer, 1973; Anderson, 2002). *Ophiostoma piceae* (Münch) Syd. & P. Syd. is one of the most important sapstain fungi, but this damage can be easily controlled by rapid drying or chemical treatment. Public concerns have encouraged a search for non-chemical methods for sapstain prevention. Biocontrol of wood sapstain fungi has attracted considerable interest (Morrell and Sexton, 1990; Behrendt *et al.*, 1995; Ejechi, 1997; Kay, 1997; Sutherland and Brasier, 1997; Schoeman *et al.*, 1999; Score *et al.*, 1998; White-McDougall *et al.*, 1998; Brown and Bruce, 1999; Brown *et al.*, 1999; Smouse *et al.*, 1999; Yang and Rossignol, 1999; McAfee *et al.*, 2001). *Trichoderma harzianum* Rifai is a potential biocontrol agent against *O. piceae*; however, it has not been commercialized due to its highly variable field performance (Freitag *et al.*, 1991). The reasons for these failures are

difficult to determine due to the inability to resolve individual fungal hyphae in a fungal consortium within the wood.

Fungal hyphae containing chitin can be visualized in wood sections using fluorescent coupled lectins, but these materials do not allow differentiation of individual species (Morrell *et al.*, 1985; Xiao *et al.*, 2000). This artificial staining method is also less effective on young hyphae (Xiao *et al.*, 2003a), and has not been applied to stain fungi in a wood block directly. As an alternative, a green fluorescent protein (GFP) technique allows *in situ* visualization of specific microorganisms and has been used to study interactions between *T. harzianum* and *O. piceae* in wood (Xiao *et al.*, 2003a; Xiao *et al.* 2003b; Chapter 5).

Prior establishment of *T. harzianum* effectively limited colonization of *O. piceae* on Douglas-fir sapwood (Xiao *et al.*, 2003b). In a previous study, *O. piceae* and *T. harzianum* spores were mixed together at different ratios and inoculated on wood. *O. piceae* growth decreased as the ratio of *T. harzianum* to *O. piceae* spores increased, suggesting that increasing the initial quantities of *T. harzianum* spores may enhance its biocontrol efficacy (Chapter 5). Metabolites produced by *T. harzianum* have antifungal properties and have been used for the control of wood degrading fungi (Bruce *et al.*, 1984; Claydon *et al.*, 1987; Bettucci *et al.*, 1988; Avent *et al.*, 1992; Srinivasan *et al.*, 1992; Horvath *et al.*, 1995; Bruce *et al.*, 1996; Hill *et al.*, 1997), however, it is difficult to determine if these metabolites play a role in biocontrol of *O. piceae*. One approach to determine the role of metabolites is to inoculate *O. piceae* to wood that has been colonized by *T. harzianum* which is

killed prior to *O. piceae* addition. Toxic *T. harzianum* metabolites should still provide protection to wood while failure to protect would suggest that nutrient competition or mycoparasitism played more important roles. In this report, we describe tests to assess the effect of prior colonization by *T. harzianum* on subsequent *O. piceae* growth.

MATERIALS AND METHODS

O. piceae isolate AU135-1 isolated from white spruce was kindly provided by Dr. Adnan Uzunovic at Forintek, Canada Corp. *T. harzianum* isolate 15B isolated from Douglas-fir roots was supplied by the U.S.D.A. Forestry Science Laboratory in Corvallis, Oregon. These fungi were transformed with a *gfp* gene as previously described (Xiao *et al*, 2003a). Isolates OPGF-I and THGF-I were two of the *gfp* transformants produced from *O. piceae* and *T. harzianum*, respectively. Freshly sawn Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) sapwood lumber was cut into wood blocks (10 × 20 × 40 mm) and sterilized by exposure to 2.5 Mrads of ionizing radiation from a cobalt 60 source.

Fungal Inoculation

Two hundred twenty wood blocks were dipped into non-transformed *T. harzianum* spore suspension (1×10^7 /ml) and incubated for 0, 2, and 4 d. Sixty-eight wood blocks without any treatment served as the control blocks. The colonized blocks were either used directly or the fungus was killed by either gamma irradiation (γ -irradiation) or autoclaving. After receiving one of these treatments, blocks were inoculated with a *gfp* transformed *O. piceae* OPGF-I spore suspension (1×10^7 /ml). Each treatment was tested on three replicates (Table 6.1).

Table 6.1: Treatments used to assess the effectiveness of live or killed *T. harzianum* against *O. piceae*

Notation	<i>T. harzianum</i> inoculation	Killing of <i>T. harzianum</i>
Control-NS ¹	None	Not killed
Control-GS ¹	None	γ -irradiation
Control-AS ¹	None	Autoclaving
TH0d-NS	0 d before <i>O. piceae</i>	Not killed
TH0d-GS	0 d before <i>O. piceae</i>	γ -irradiation
TH0d-AS	0 d before <i>O. piceae</i>	Autoclaving
TH2d-NS	2 d before <i>O. piceae</i>	Not killed
TH2d-GS	2 d before <i>O. piceae</i>	γ -irradiation
TH2d-AS	2 d before <i>O. piceae</i>	Autoclaving
TH4d-NS	4 d before <i>O. piceae</i>	Not killed
TH4d-GS	4 d before <i>O. piceae</i>	γ -irradiation
TH4d-AS	4 d before <i>O. piceae</i>	Autoclaving

¹NS: no extra sterilization. GS: extra sterilization by γ -irradiation, AS: extra sterilization by autoclaving.

Assessment

Surface examination of green fluorescent hyphae

Fungal colonization on block surfaces was observed using a Nikon Elipse E400 microscope equipped with a green fluorescence filter set (excitation: 465-495 nm, emission: 515-555 nm) over a seven-day period. Five spots were specified on each sample by using a needle to drill small dimples on the upper block surface (Figure 6.1). This permitted repeated examination of the same fields. The numbers of spores, germlings, and synnemata developing in each of the five 2mm diameter fields were counted under a 10× objective lens. Hyphal development was also rated based on the percentage of wood cell coverage.

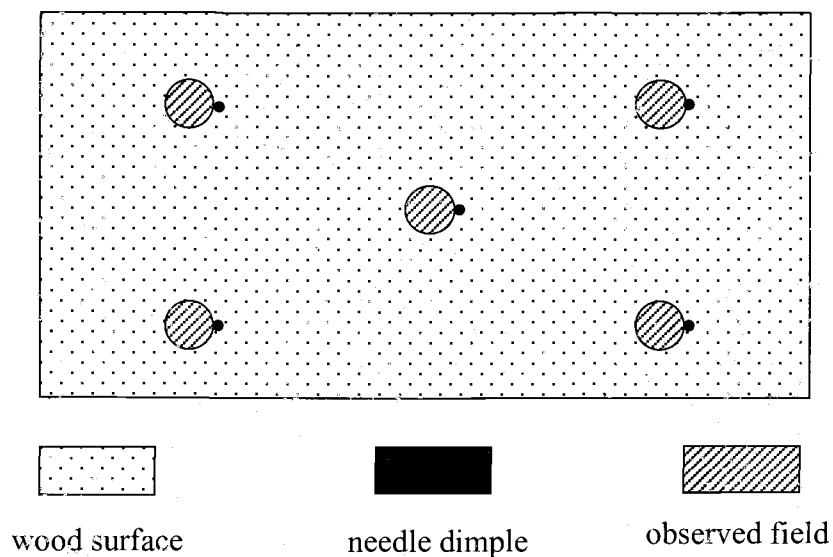


Figure 6.1: Locations of fixed observation fields (2mm diameter with a 10× objective lens) on wood blocks inoculated with OPGF-I.

Wheat germ agglutinin (WGA) fluorescent staining of wood block surfaces

After the above observations, the same wood blocks were fixed in 2% paraformaldehyde for 10 min, and washed 3 times for 10 min each in distilled water. The block surfaces were then stained with Rhodamine-conjugated wheat germ agglutinin (WGA) fluorescent stain (Molecular Probe, Eugene, USA) for 30 minutes. The block surfaces were rinsed 3 times for 15 min each in distilled water, and then fungal growth was assessed in observation fields on each block. The complete design is presented as a flow chart in Figure 6.2.

Data Analysis

O. piceae spore germination, hyphal development, and synnemata development were compared using Tukey's HSD multiple comparison method on SAS (SAS ver 8.2, SAS Institute, Cary, NC).

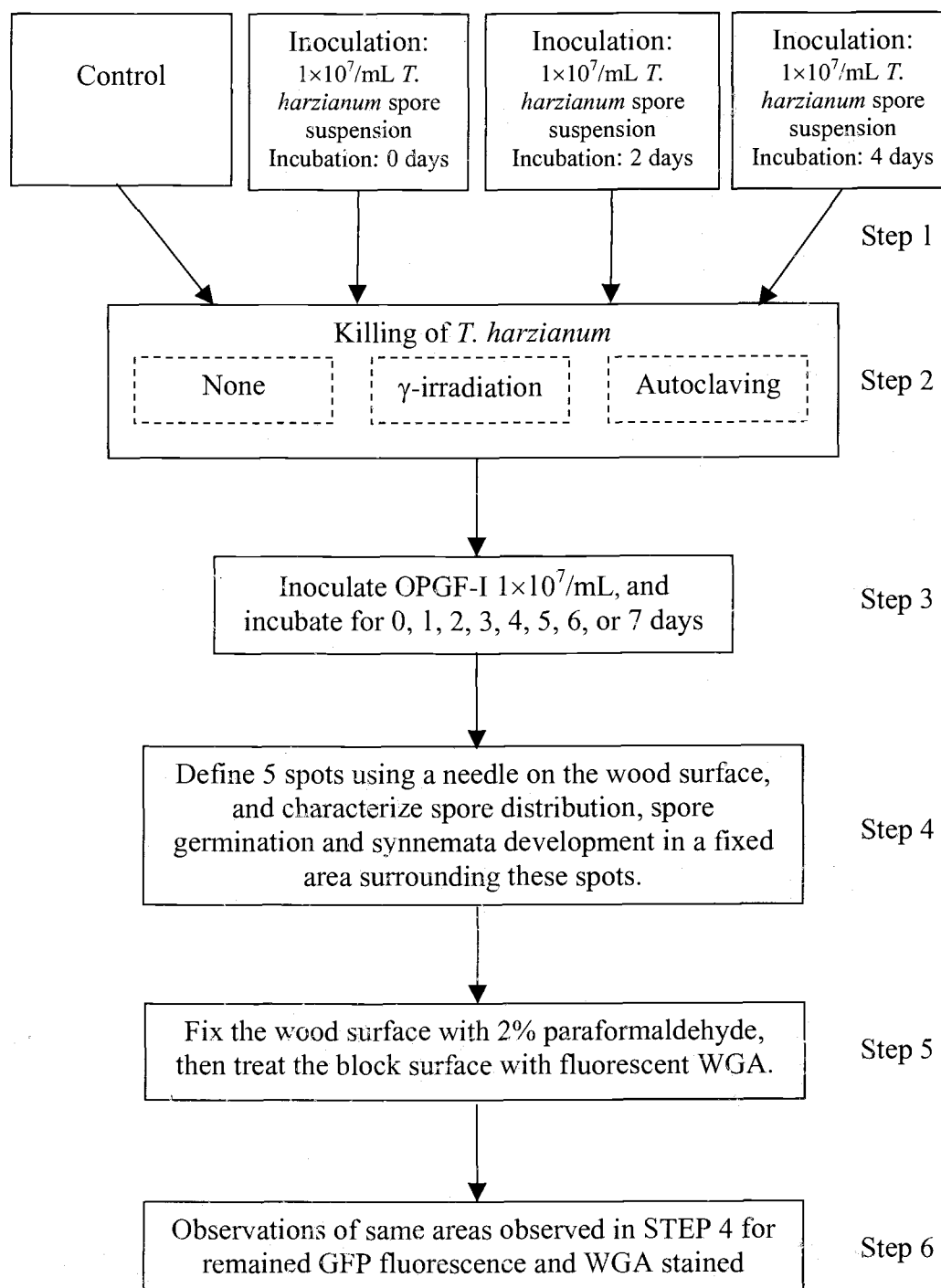


Figure 6.2: Flow chart showing experimental design used to assess the effect of live or killed *T. harzianum* on *O. piceae*.

RESULTS AND DISCUSSION

WGA Staining

Inconsistent results were obtained with WGA staining, which is specific for n-acetyl glucosamine in chitin in fungal hyphae (Morrell *et al.*, 1985; Xiao *et al.*, 2000). Lectins were less effective for staining hyphae on most wood block surfaces. Wood cells strongly autofluoresce across a wide wavelength range and this background fluorescence requires strong fluorescence in fungal hyphae for them to be visualized on a wood block surface. It is also difficult to react the hyphae with lectin then rinse excessive lectin from the wood. A second reason for the inability of lectins to react may be that all observations were made at the early growth stages when chitin production may have been insufficient for effective WGA staining. Observations of thin wood sections cut from WGA stained wood surfaces showed that faintly fluorescent hyphae were visible using the red filter but were not observed on wood block surfaces (data not shown).

It was interesting to note that WGA only allowed visualization of some *T. harzianum* germlings/hyphae on some γ -irradiated blocks (TH2d_GS and TH4d_GS) (Figure 6.3). WGA stained *T. harzianum* germlings/hyphae were not observed on a majority of TH2d_GS and TH4d_GS treated blocks, further illustrating the inconsistency of WGA for visualizing fungi on the wood block surface.

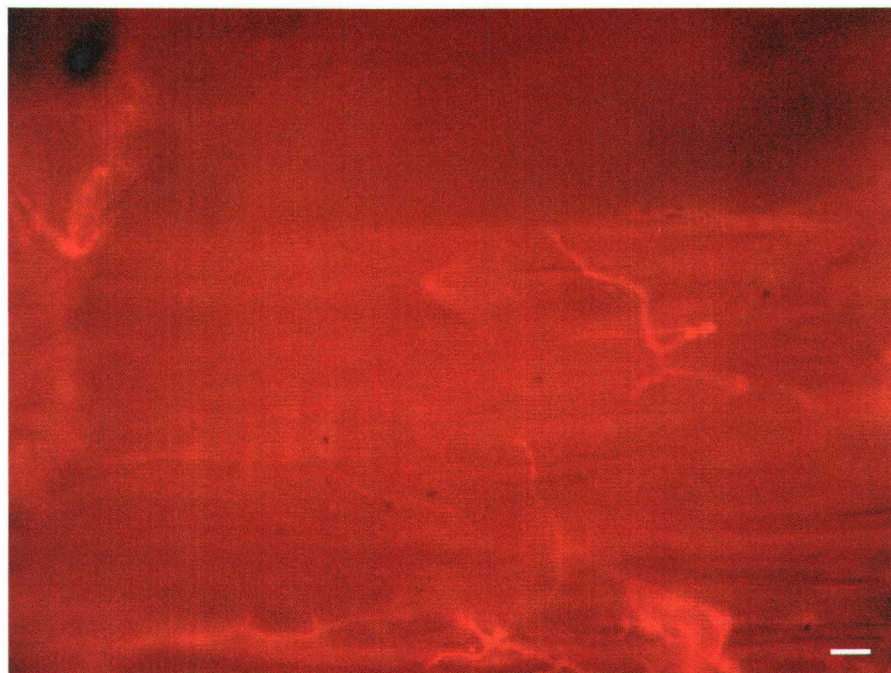


Figure 6.3: WGA stained *T. harzianum* germlings killed with γ -irradiation 2 days after inoculation on Douglas-fir sapwood (Bar: 20 μ m).

Spore Wash-off

It was difficult to observe spores of *gfp* transformed *O. piceae* immediately after inoculation due to strong autofluorescence from the wood block surface, although spores can be viewed easily under a fluorescence microscope using a dark background.

O. piceae spores became visible 2-4 hours after inoculation on blocks not sterilized after *T. harzianum* growth (NS treatment), indicating the initiation of spore activity. Spores tended to be washed from the wood surface after WGA staining (Figure 6.4). Approximately 60% of the spores were washed away during WGA staining, although up to 90% of spores were lost from some blocks (Figure

6.5). Germlings were well developed on control_NS and TH0d_NS treated blocks. Spores became visible on TH2d_NS and TH4d_NS treated blocks, but failed to germinate, suggesting a fungistatic effect by pre-established *T. harzianum*.

O. piceae spores on blocks that were sterilized after *T. harzianum* colonized (γ -irradiation or autoclaving — GS or AS treatment respectively) became visible on wood 10 hrs after inoculation. Slower spore germination compared to the non-sterilized blocks may reflect the alterations in nutrient condition or release of antibiotics caused by sterilization. No germlings were observed on sterilized blocks 24 hrs after inoculation. The average spore wash-off rate was 23% for these treatments; however, the wash-off rate was larger than 90% on some individual blocks (data not shown). This spore wash-off rate was lower than that found with non-sterile treatments obtained in the first few hours after inoculation and the results were more uniform (Figure 6.4). This implies that spores require time to attach firmly to the wood surface, and that sterilization may alter attachment ability.

Spore germination is a critical stage in the life cycle of a microorganism where the microorganism is most vulnerable to environmental factors. Despite its importance in colonization, spore germination on wood-based systems has received only limited study (Morton and French, 1966; Toole, 1971; Schmidt and French, 1979; Przybylowicz and Corden, 1986; Kreber, 1991). Spore germination on wood has typically been observed using some form of artificial staining. Rinsing to remove the stains may inadvertently dislodge spores from the wood

surface, resulting in fewer spores available for observation and potentially altering the resulting conclusions from these studies. GFP allows *in situ* monitoring of spore germination without artificial staining, reducing the risk of spore loss and allowing examination of fungal responses in the early growth stages.

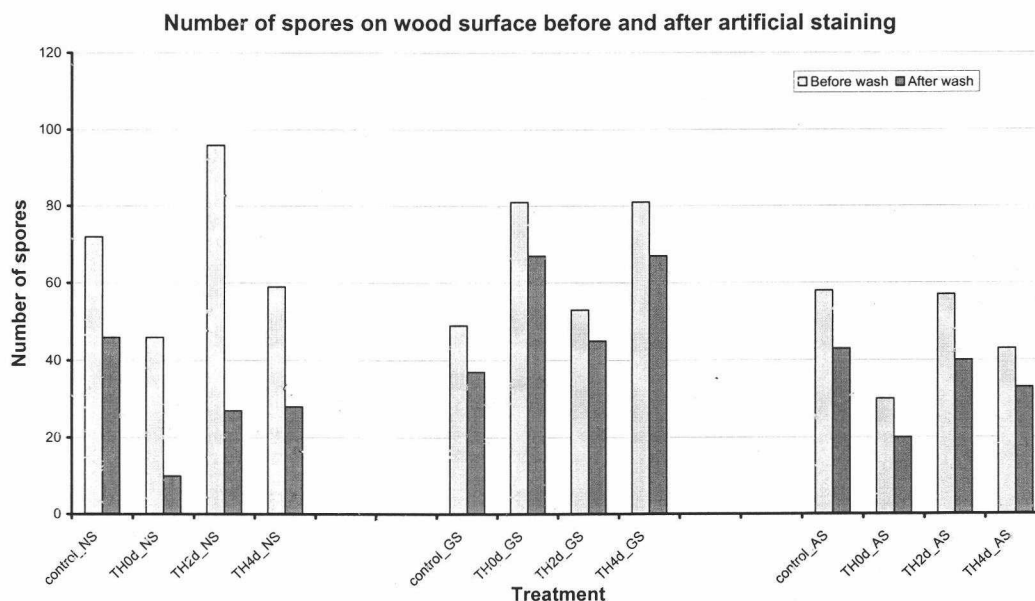


Figure 6.4: Number of *O. piceae* spores on wood surfaces before and after staining with Rhodamine-coupled wheat germ agglutinin (WGA). Each column represents the mean of observations from 15 fields. For NS treatment: washing took place 4 hrs after inoculation; for GS and AS treatments: washing took place 24 hrs after inoculation.

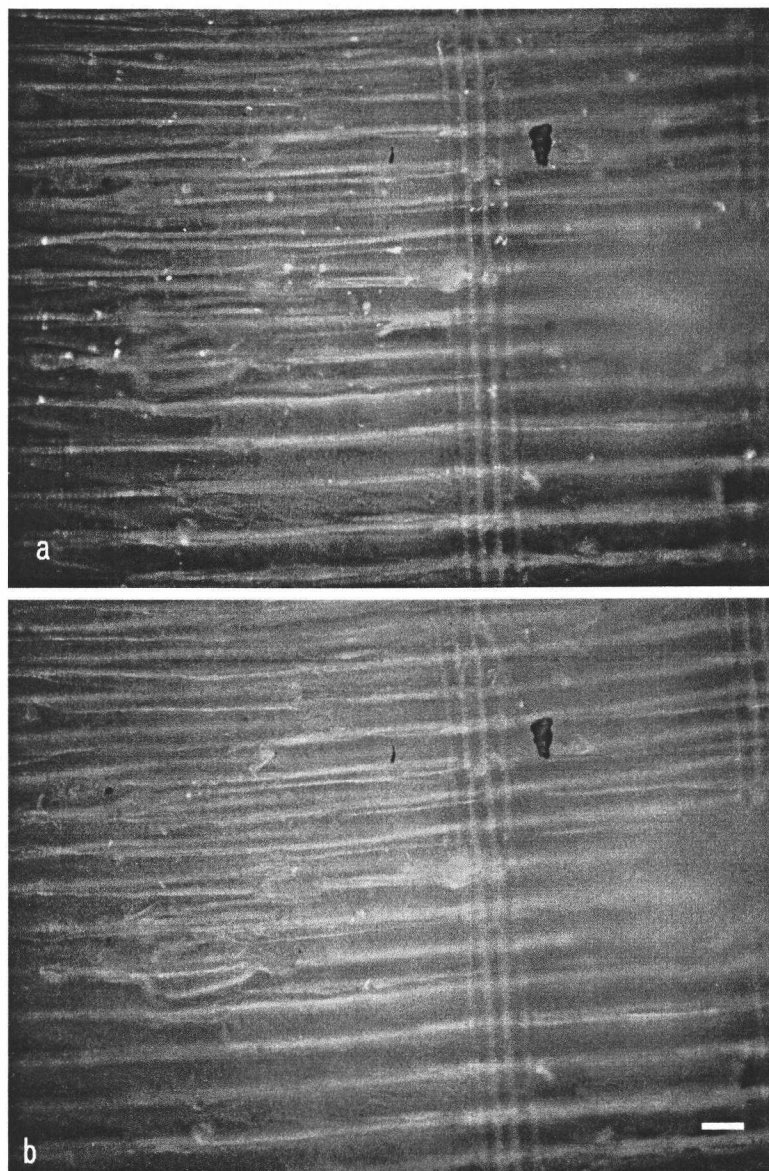


Figure 6.5: An example of spores on wood surfaces: a) before and b) after artificial staining showing nearly 90% loss in spores due to rinsing (Bar: 50 μ m).

Spore Germination

Spores germinated rapidly on control_NS and TH0d_NS treated blocks, and germlings were well-developed 24 hrs after inoculation. Spore germination

was significantly lower on TH2d_NS and TH4d_NS treated blocks, suggesting that prior establishment of *T. harzianum* effectively inhibited *O. piceae* spore germination, a finding that is consistent with previous results (Figure 6.6) (Table 6.2) (Xiao *et al.*, 2003b). Complete spore germination was not observed 24 hrs after inoculation on blocks that were sterilized after *T. harzianum* inoculation, but it began at this stage and was observed 48 hrs after inoculation on blocks. γ -irradiation and autoclaving affect fungal growth differently for different fungal species (Morton and French, 1966; Byrne and Minchin, 1992; McAfee and Gignac, 1996; Williams *et al.*, 1998). In this study, both γ -irradiation and autoclaving had a negative effect on *O. piceae* spore germination, but the effect was more severe with autoclaving (Table 6.2).

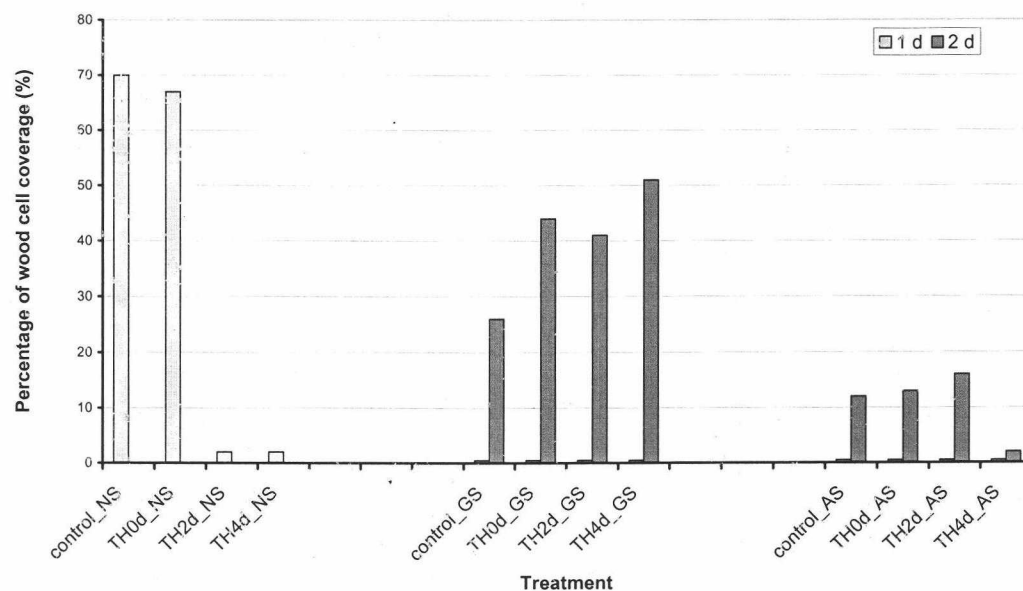


Figure 6.6: Effect of live or killed *T. harzianum* on *O. piceae* spore germination on Douglas-fir sapwood. Each column represents the mean value of three replicates.

Hyphal Development

Hyphal development was rated as the percentage of wood cell coverage by *O. piceae* hyphae (Figure 6.7).

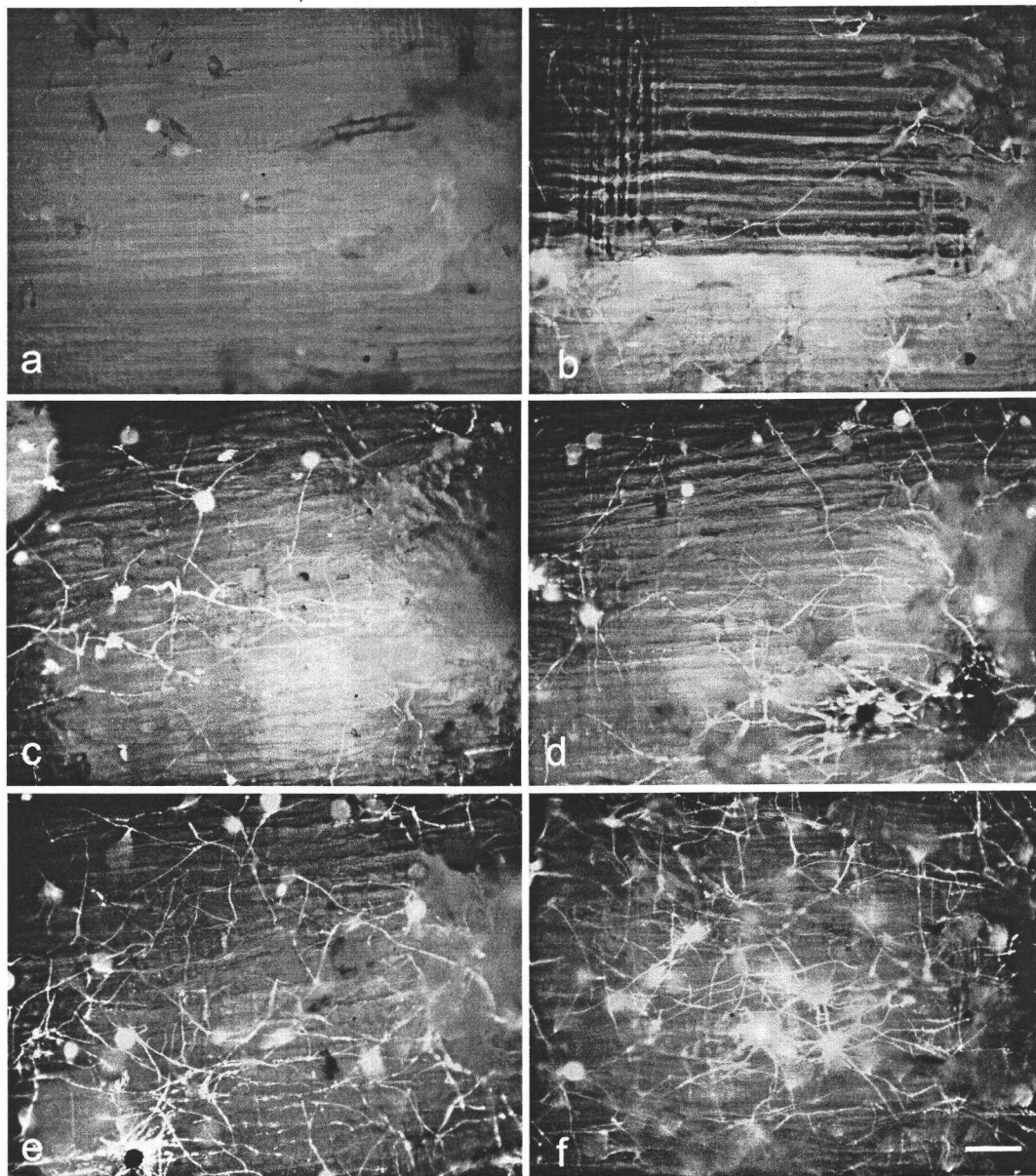


Figure 6.7: Representative micrographs showing selected degrees of hyphal coverage: a) <1% b) 10% c) 30% d) 50% e) 70% or f) 90% (Bar: 100 μ m).

Table 6.2: Tukey's HSD analysis results for *O. piceae* spore germination, hyphal and synnemata development on Douglas-fir sapwood

Spore Germination	Hyphal Development	Synnemata Development
Control-NS TH0d-NS <i>a</i> TH4d-GS TH0d-GS <i>b</i> TH2d-GS <i>c</i> Control-GS <i>d</i> TH2d-AS TH0d-AS Control-AS TH4d-AS TH4d_NS <i>e</i> TH2d_NS	Control-NS TH0d-NS <i>a</i> TH0d-GS TH2d-GS <i>b</i> TH4d-GS <i>c</i> Control-GS <i>d</i> Control-AS <i>e</i> TH0d-AS TH2d-AS TH2d_NS <i>f</i> TH4d_NS TH4d-AS	Control-AS TH0d-AS TH0d-GS TH2d-AS TH4d-GS <i>a</i> Control-GS TH0d-NS TH2d-GS TH4d-AS <i>b</i> Control-NS TH2d-NS TH4d-NS <i>c</i>

¹Treatments with the same letter are not significantly different (at a 0.05 significant level). The means for treatments are in a decreasing order from above to below.

As with spore germination, hyphal development of *O. piceae* was inhibited by live *T. harzianum* (Figure 6.8), and was affected negatively by γ -irradiation or autoclaving (Table 6.2). *T. harzianum* killed by γ -irradiation after prior establishment did not limit hyphal development of *O. piceae*. Killing *T. harzianum* by autoclaving resulted in inhibition of *O. piceae* growth on TH4d_AS treated blocks, but not on TH2d_AS treated blocks. These observations were similar to those obtained in the spore germination tests. The inability of killed *T. harzianum* to protect wood against *O. piceae* on all but the TH4d_AS treatment suggests that

T. harzianum might inhibit fungal growth by producing toxic metabolites. If nutrient depletion was the dominant mode of action, then *O. piceae* growth should have been inhibited on TH4d_GS treated blocks since established *T. harzianum* should have depleted readily available nutrients.

Metabolites produced by *T. harzianum* have antifungal properties and have been used for the control of wood degrading fungi. Killing *T. harzianum* using γ -irradiation may have kept hyphal cells intact. As a result, toxic metabolites may not have been released. Autoclaving produces massive damage to *T. harzianum* cells, potentially releasing metabolites. *T. harzianum* spore germination was spotty 2 days after inoculation, and may have been inadequate for protection of wood on TH2d_AS treated blocks.

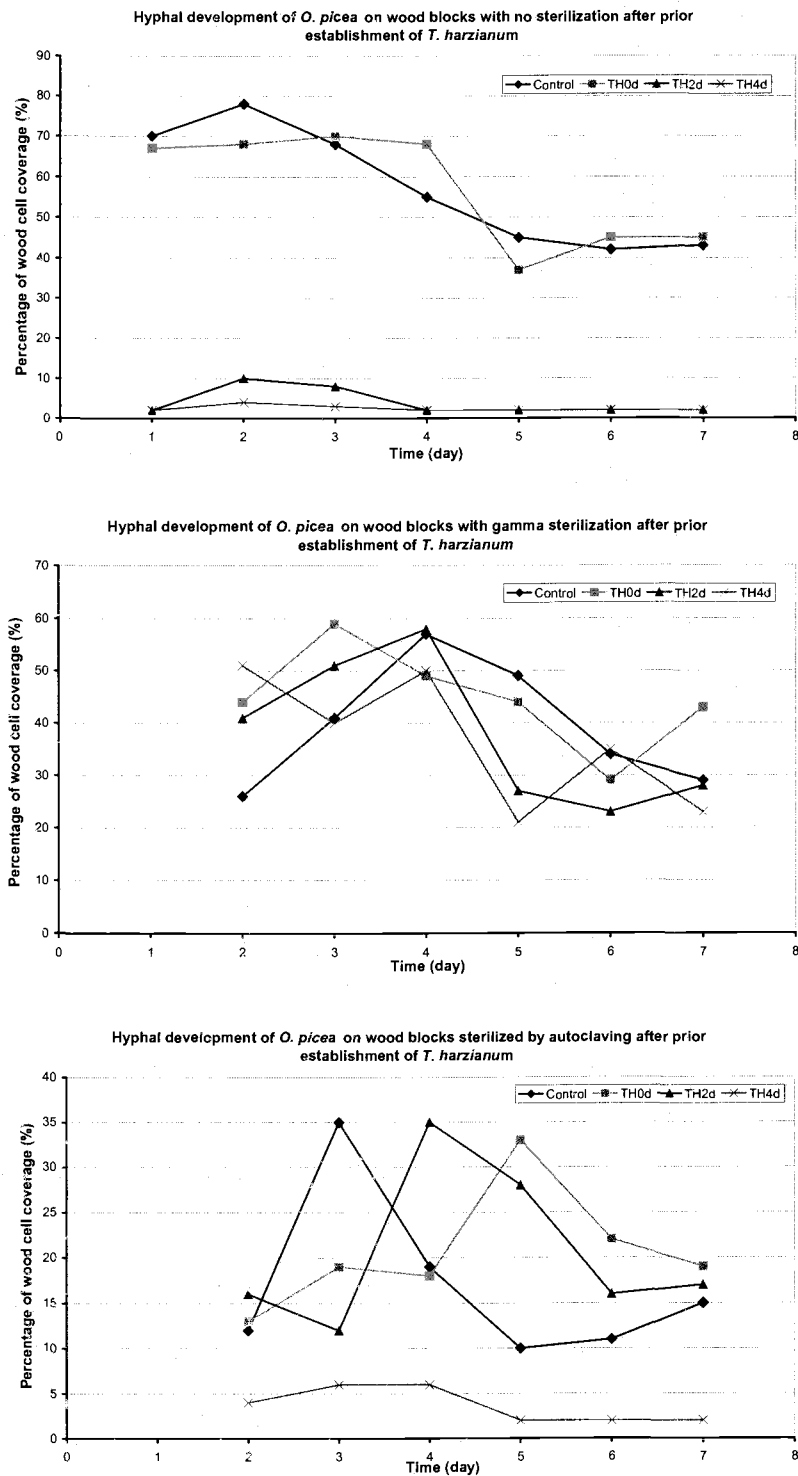


Figure 6.8: *O. piceae* hyphal development on Douglas-fir sapwood blocks with living or killed *T. harzianum* (mean value of 3 replicates).

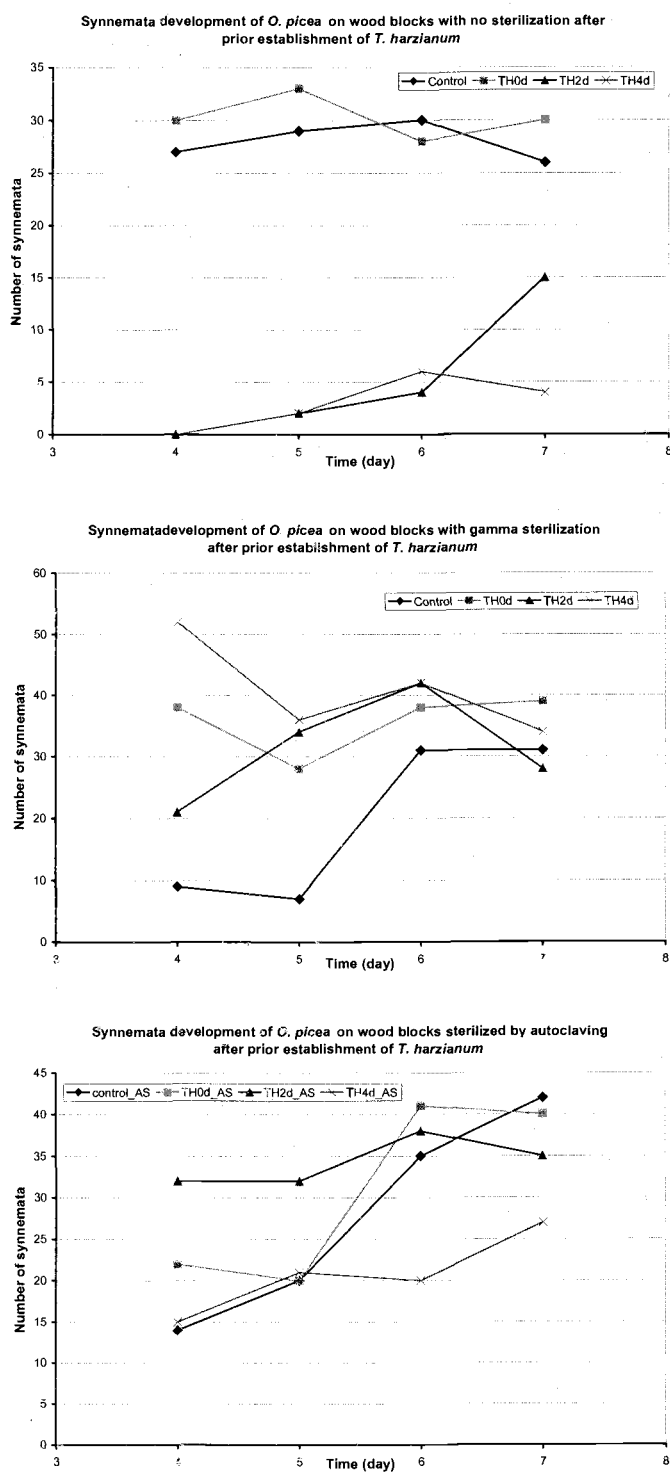


Figure 6.9: *O. piceae* synnemata development (with spore heads on) on Douglas-fir sapwood blocks with living or killed *T. harzianum* (mean value of 3 replicates).

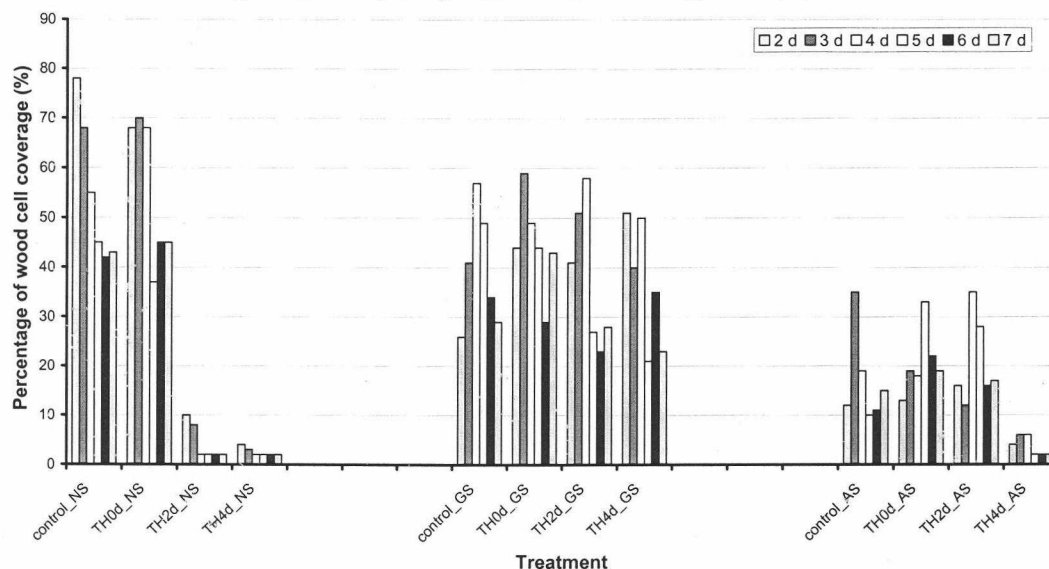
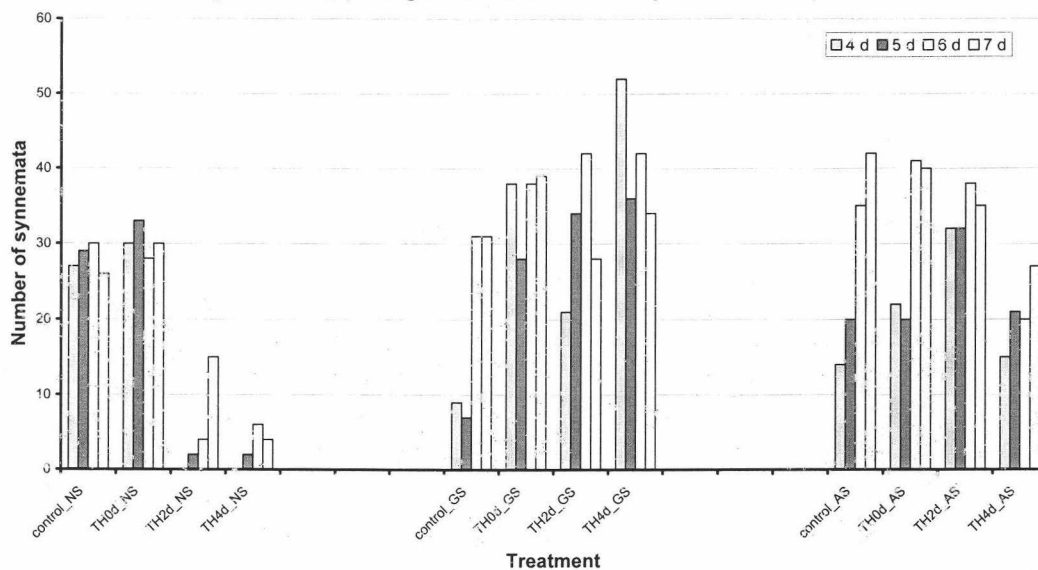
Synnemata Development

Synnemata development of *O. piceae* was significantly retarded by live *T. harzianum* (Figure 6.9). However, *O. piceae* synnemata developed regularly on all wood blocks with killed *T. harzianum* with no significant difference between treatments (Table 6.2). Previous studies showed that *O. piceae* developed synnemata and spore heads under stress, even when hyphal development was limited (Xiao *et al.*, 2003b; Chapter 5). This may explain why the TH4d_AS treatment inhibited *O. piceae* hyphal growth but not synnemata development.

Effects of Sterilization on *O. piceae* Growth

Both γ -irradiation and autoclaving negatively affected *O. piceae* spore germination and hyphal growth, although autoclaving had the greatest effect (Table 6.2) (Figure 6.5). Synnemata development was unaffected by sterilization (Table 6.2) (Figure 6.10). Decreases in spore germination and hyphal development may be due to the alteration of nutrients after γ -irradiation and autoclaving. However, the remaining nutrients may still be sufficient to allow for normal *O. piceae* synnemata development.

No evidence of cell lysis or morphological changes were observed in *O. piceae* cells in any of the treatments in this study, suggesting that mycoparasitism was not likely to be a dominant mode of action.

Figure 6.10a: *O. picea* growth on wood surface: hyphal developmentFigure 6.10b: *O. picea* growth on wood surface: synnemata developmentFigure 6.10: Effect of live or dead *T. harzianum* on *O. piceae*: a) hyphal and b) synnemata development on Douglas-fir sapwood (mean value of 3 replicates).

CONCLUSIONS

Fluorescent coupled lectin staining was less useful for studying spore germination and early hyphal growth of fungi on wood. *T. harzianum* and *O. piceae* appeared to be antagonistic to each other. Prior establishment of *T. harzianum* effectively inhibited *O. piceae* growth on freshly sawn Douglas-fir sapwood, but the inhibition was lost when *T. harzianum* was killed by γ -irradiation. The results suggest that nutrient depletion was unlikely to be the main mode of action of *T. harzianum*. Killing *T. harzianum* by γ -irradiation did not appear to affect the integrity of fungal cells, suggesting that any toxic metabolites from *T. harzianum* would remain inside the hyphae. On the other hand, *T. harzianum* killed with autoclaving produced partial protection against *O. piceae* when *T. harzianum* was well established before being killed. The results suggested that *T. harzianum* must remain alive to be most active against *O. piceae*.

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Chapter 7: Conclusions

CONCLUSIONS

While microbial colonization of wood is presumed to be characterized by a myriad of interactions between numerous organisms, studying these processes is often difficult owing to the opaque nature of the wood and the inability to readily distinguish among the many species colonizing the material. Fungal hyphae containing chitin in wood sections can be visualized using fluorescent coupled lectins, but these materials do not allow individual species to be differentiated, and were not useful in studying spore germination and early growth of fungi on wood block surfaces. One method for enhancing the ability to distinguish organisms is to induce specific proteins in one or more organisms that can be detected using fluorescence or other light microscopic techniques. The insertion of genes for the production of green fluorescent proteins produced by the jellyfish, *Aequora victoria*, has been widely used to visualize a variety of organisms. In this study, an important wood sapstain fungus, *Ophiostoma piceae*, and its biocontrol agent, *Trichoderma harzianum* were transformed using a green fluorescent protein (GFP) gene under the control of the *ToxA* promoter of *Pyrenophora tritici-repentis*.

An important first step in this transformation is to develop systems for eliminating the fungal cell wall so that the genes can be inserted into the cells. The simplest method for accomplishing this task is to generate fungal protoplasts. Protoplasts are the wall-less states of cells after the cell walls have been removed

through enzymatic digestion. The degree of cell wall removal relates to the success in transformation and the fungal regeneration oppositely. An optimum protoplasting procedure was developed for *O. piceae* in Chapter 2. Protoplasts could be produced from 20-hour-old germlings of *O. piceae* using a MgSO_4 -maleic-NaOH buffer. Higher enzyme concentrations and treatment times increased protoplast release but reduced the ability of the protoplasts to regenerate. Exposure to 0.7% lytic enzyme for 30 minutes appeared to produce optimum release and regeneration for the conditions evaluated.

The pCT74 vector appeared to effectively transform both *O. picea*, and *T. harzianum* under the control of the *ToxA* promoter of *Pyrenophora tritici-repentis* (Chapter 3). Growth of transformants was similar to the non-transformed strains for both species. Transformation did not adversely affect the ability of either fungus to colonize wood, cause discoloration or sporulate. Surface growth of the transformed fungi on wood was easily monitored *in situ*. The resulting transformants should prove useful for assessing the growth of young hyphae in wood, although the use of thin sections to study internal growth patterns may be problematic.

Biocontrol uses one microorganism to inhibit the detrimental activities of another. Being environmentally benign, biocontrol of wood sapstain fungi using *T. harzianum* has attracted many research interests, but no commercialized application has been reported so far due the high variation in its field performances. *T. harzianum* spore germination was slower than that of *O. piceae*

on freshly sawn Douglas-fir sapwood, while the spore germination of two fungi was comparable in artificial medium. This may partially explain the highly variable field performances in *T. harzianum* as a biocontrol agent.

In Chapter 4, the effect of prior establishment of *T. harzianum* on *O. piceae* growth on Douglas-fir wood was examined. OPGF-I, *gfp* transformed *O. piceae* strain, was inoculated after *T. harzianum* grew on wood 0, 2, 4, 6 or 8 days. Although *T. harzianum* spores germinated more slowly than those of *O. piceae* on fresh Douglas-fir sapwood, the former fungus colonized the wood more rapidly once it germinated. Simultaneous inoculation or slight delays between *T. harzianum* and *O. piceae* introduction allowed the stain fungus to grow normally. *O. piceae* growth was limited when *T. harzianum* was well established, while *T. harzianum* was unable to sporulate on wood when *O. piceae* was inoculated at the same time. These results suggest that both fungi were antagonistic to one another. The partial protection and importance of internal colonization by the biocontrol fungus partly explain the failures reported in field tests and suggest the need for further trials to better understand the role of initial colonization on biocontrol performance.

As described in Chapter 4, prior establishment of *T. harzianum* appeared to increase the effectiveness of this fungus against *O. piceae*. *O. piceae* and *T. harzianum* are antagonistic to each other, and the species that germinates first will tend to dominate the substrate. One approach to enhance successful *T. harzianum* colonization may be to apply an overwhelming number of spores producing toxic

substances. In Chapter 5, freshly sawn Douglas-fir wood blocks were inoculated with a spore suspension containing spores of a *gfp* transformed *O. piceae* and a non-transformed *T. harzianum* at different ratios. The ratio of *T. harzianum* to *O. piceae* spores had a positive effect on the biocontrol efficacy of *T. harzianum*. Inoculum containing 10:1 *T. harzianum* : *O. piceae* (TH:OP) inhibited internal growth of *O. piceae*, but did not limit the surface hyphal growth or synnemata development. *T. harzianum* inhibited both superficial and internal *O. piceae* growth when the TH:OP ratio was 100:1 or higher. These results suggest that biocontrol efficacy may be improved by increasing the spore concentration in the *T. harzianum* inoculum or by periodically reapplying *T. harzianum* spore suspensions.

Metabolites produced by *T. harzianum* have antifungal properties and have been used for the control of wood degrading fungi; however, it is difficult to determine if these metabolites play a role in biocontrol of *O. piceae*. One approach to determine the role of metabolites is to inoculate *O. piceae* on wood that has been colonized by *T. harzianum* which is killed prior to *O. piceae* addition. Toxic *T. harzianum* metabolites should still provide protection to wood while failure to protect would suggest that nutrient competition or mycoparasitism played more important roles. In Chapter 6, we described tests to assess the effect of live and dead *T. harzianum* on *O. piceae* growth. Prior establishment of *T. harzianum* effectively inhibited *O. piceae* growth on freshly sawn Douglas-fir sapwood, but the inhibition was lost when *T. harzianum* was killed by γ -irradiation. The results

suggest that nutrient depletion was unlikely to be the main mode of action of *T. harzianum*. Killing *T. harzianum* by γ -irradiation did not appear to affect the integrity of fungal cells, suggesting that any toxic metabolites from *T. harzianum* would remain inside the hyphae. On the other hand, *T. harzianum* killed with autoclaving produced partial protection against *O. piceae* when *T. harzianum* was well established before being killed. The results suggested that *T. harzianum* must remain alive to be most active against *O. piceae*.

In conclusion, GFP is proven to be useful in studying fungal interactions in wood. It may help understanding of the fundamentals in biocontrol of wood degrading microorganisms, which may lead to identification of effective biocontrol strategies.

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